

PULMONARY INTRAVASCULAR MACROPHAGES IN THE RABBIT

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ABSTRACT

Pulmonary intravascular macrophages (PIMs) promote lung inflammation and are found in ruminants, horses, pigs, cats, and dolphins, but not in primates, rats and mice. Rabbits are used to study mechanisms of lung inflammation in humans, but disagreement exists whether rabbits have PIMs. This study examined rabbits for PIMs, and their influence on endotoxin-induced lung inflammation.

Rabbits were treated with gadolinium chloride (10 mg/kg intravenous: Group GC, $n=6$) to produce apoptosis in PIMs, or with saline (Group SAL, $n=6$). Rabbits were euthanized 48 hours later. Light microscopic examination of epoxy-embedded rabbit lung sections revealed mononuclear phagocytes in alveolar septa. Transmission electron microscopy confirmed PIMs with lysosomes and close attachment to capillary endothelium. Light microscopic immuno-cytochemistry using rabbit anti-macrophage antibody (RAM-11) showed staining of septal and alveolar macrophages. There was no difference in number of RAM-11 positive septal cells between SAL and GC rabbits ($P=0.2$).

Rabbits were administered intravenous *E.coli* 0127:B8 endotoxin (100 $\mu\text{g/kg}$) 48 hours after GC (GC-LPS; $n=5$) or SAL treatment (SAL-LPS; $n=6$), and euthanized 24 hours later. Rabbits in both LPS treated groups were hypocalcaemic and exhibited compensated metabolic acidosis compared to SAL rabbits. Four rabbits died in the SAL-LPS group within 24 hours of the endotoxin treatment and were replaced. None died in the GC-LPS group (Chi-square comparison for survival $P=0.063$). Greater numbers of septal heterophils were found in groups SAL-LPS and GC-LPS compared to SAL and GC. $\text{TNF}\alpha$ protein in serum, and IL-1 β and IL-6 mRNA in lung tissues were increased in SAL-LPS compared to SAL and GC rabbits. Lung tissues from SAL-LPS rabbits but not in GC-LPS showed moderate inflammation, but lung wet/dry ratios were not different. Lung tissue $\text{TNF}\alpha$, IL-1 β and IL-6 mRNA, myeloperoxidase activity, and serum $\text{TNF}\alpha$ were reduced in GC-LPS animals compared to SAL-LPS. Immuno-electron microscopy revealed $\text{TNF}\alpha$ in PIMs in normal and LPS-treated rabbits. Lung and liver tissue $\text{TNF}\alpha$, IL-8 and MCP-1 protein concentrations were not different between groups. GC did not appear to reduce liver inflammation. These data show that rabbits have low numbers of

PIMs. GC treatment induced apoptosis in PIMs and reduced endotoxin-induced lung inflammation and mortality.

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DEDICATION

I would like to dedicate this thesis to my husband, Brian A.A. Novakovski for believing in me, and to my wonderful sons: Stephen Ian and Andrew Hugh.

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LIST OF ABBREVIATIONS

ALI: Acute Lung Injury

ARDS: Acute respiratory distress syndrome

BAL: Bronchoalveolar lavage

CCL: CC chemokine ligand

CTAC: Cetyltrimethylammonium Chloride

CXCL: CXC chemokine ligand

DAP: Diastolic arterial blood pressure

EDTA: Ethylenediaminetetraacetic Acid

ELISA: Enzyme Linked Immunoabsorbent Assay

FI: Fraction of Inspired

GC: Gadolinium chloride

HBSS: Hanks Balanced Salt Solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

I κ B: Nuclear Factor of κ light polypeptide gene enhancer in B-cells inhibitor

IL: Interleukin

IRAK: Interleukin-1 Receptor-Associated kinase

JNK: c-Jun N-terminal Kinase

LPS: Lipopolysaccharide

MAP: Mean arterial blood pressure

MAP kinase: Mitogen Activated Protein kinase

My-D88: Myeloid Differentiation primary response gene (88)

MPO: Myeloperoxidase

NO: Nitric oxide

NF κ B: Nuclear Factor κ light-chain enhancer of activated B cells

PBS: Phosphate Buffer Solution

PIM: Pulmonary Intravascular Macrophage

RT-PCR: Reverse Transcriptase – Polymerase Chain Reaction

SAP: Systolic arterial blood pressure

TMB: 3', 5, 5'- tetramethylbenzidine hydrochloride

TNF: Tumour Necrosis Factor

TRAF-6: TNF Receptor Associated Factor

INTRODUCTION

1.1 Inflammation

Inflammation is the response of the body to invasion of pathogens, antigens and/or physical or thermal injury. Not only does the response involve removing or rendering safe the body from invasion or injury, but also promotes healing and recovery. There are times however, when the pathogen invasion or injury overwhelms, and the inflammatory response is not sufficient to remove pathogens or provide repair. The inflammatory response itself can also overwhelm, and become detrimental to the body, for example, during sepsis or acute lung injury. Both situations can lead to the demise of the individual. Therefore, tight regulation of the inflammatory response tailored to the type of insult is important for a successful outcome.

Although there is a great deal of overlap between events, the four stages are traditionally described as: 1) Destruction and removal of injurious agents; 2) Containment of the harmful agents; 3) Stimulation and amplification of the immune response and 4) Promotion of healing (Botting and Botting, 2000). Cells of the immune system are involved in this process. Macrophages are considered to be tissue sentinel cells which ingest invading pathogens, process antigens and are involved in antigen presentation to T and B lymphocytes. Neutrophils are recruited to the site of inflammation with release of chemoattractants (chemokines) and also ingest bacteria. Cytokines are used to communicate between cells and mediate the process of inflammation. Cytokines are considered to have pro- or anti- inflammatory functions, acting on the cells that produce them, and also on neighbouring cells (autocrine and paracrine actions). Endocrine actions are also important as cytokines are transported throughout the body using the circulatory system. Cytokines bind to cell surface receptors and initiate internal signalling pathways which then influence gene transcription. In this way, the inflammatory process is up- or down- regulated. For example, among many pro-inflammatory actions, tumour necrosis factor- α (TNF- α) can upregulate the production of nitric oxide (NO) causing vasodilatation, and interleukin (IL)-10 has anti-inflammatory actions by limiting the production of TNF α . The same cytokines can have different actions on different cells

with changing circumstances. Chemokines are responsible for attracting cells to the site of injury or inflammation and are produced by leukocytes and endothelial cells. Different chemokines can attract different cell types depending on their structure (Rankin, 2004). Relevant cytokines pertaining to monocyte, macrophage and neutrophil function and important to this work are described later.

The start of the inflammatory process usually stems from cell injury. Vasodilatation is produced by released mediators from disrupted cells and leads to endothelial cell cytoskeleton changes, opening pores, and causing capillary leakage. Thromboxane A₂ causes platelet aggregation at the site of injury, which is part of tissue protection and healing. Exudation of fluid into the site of injury through leaky endothelium causes swelling and dilution of toxins, and provides a fluid environment for movement of immunoglobulins and leukocytes.

Cells from the immune system are recruited from the circulation in order to assist in removal of pathogens and tissue debris. Margination of leukocytes along cytokine-activated endothelial walls occurs through production of adhesion molecules (selectins, integrins). Both IL-1 and TNF- α stimulate production of selectins which mediate leukocyte capture (P or E-selectin on endothelium and L-selectin on leukocytes). Integrins are transmembrane glycoproteins that attach cells to extracellular matrix proteins of the basement membrane, ligands or other cells, and also initiate cell signalling.

Captured leukocytes go through a transient and reversible rolling stage followed by a firmer, adhesive, slow rolling stage through CD18 integrins. Following this, migration of the leukocyte from blood vessel to tissue space occurs, a process called diapedesis. Neutrophils are not normally found in large numbers in tissue spaces. Transmigration, using chemotaxis, is important to recruit neutrophils to the point of injury or invasion. Within 24 hours of onset of inflammation, monocytes are also recruited and differentiate into macrophages. These cells clear dead neutrophils through CD31 interactions, pathogens and cell debris, and initiate healing (Rankin, 2004).

1.2 Acute Lung Injury and Endotoxin

Acute Lung Injury (ALI) and the more severe form, Acute Respiratory Distress syndrome (ARDS), result from direct lung damage, or as a result of indirect contact with endotoxin or cytokines originating elsewhere in the body. In humans, the condition is characterized by clinical features of: 1) acute onset; 2) bilateral alveolar infiltrates on radiograph with a normal left atrial blood pressure (noncardiogenic oedema); 3) increased alveolar-arterial partial pressure of oxygen gradient ($\text{PaO}_2/\text{FIO}_2$ ratio of $<300\text{mmHg}$ for ALI and $<200\text{mmHg}$ for ARDs) and 4) loss of lung compliance. The condition is increasingly recognized in veterinary medicine, and manifests as 'equine neonatal respiratory distress syndrome' and 'vetALI/ARDS' in dogs and cats (Wilkins *et al.*, 2007). The systemic inflammatory reaction of sepsis results in multiple organ dysfunction and the lung may be included in the list of organs affected. ALI/ARDS from sepsis leads to high mortality in species with or without pulmonary intravascular macrophages (PIMs), but it appears the degree of lung injury is more intense when PIMs are present (Gill *et al.*, 2008), (Parbhakar *et al.*, 2005). The involvement of PIMs in ALI/ARDS is the subject of intense research using a variety of animal models.

Lipopolysaccharide (LPS) is a glycolipid present in the outer cell wall of Gram-negative bacteria. LPS is composed of a lipid polar head group (Lipid A) and a chain of repeating disaccharides. Most of the observed response to LPS can be reproduced with lipid-A, but the disaccharide chain influences the magnitude of the response. LPS attaches to LPS-binding protein and this combination stimulates CD14 (surface membrane receptor) on monocytes and macrophages, and to a lesser extent, neutrophils (Sabroe *et al.*, 2005). Cell activation is mediated through the transmembrane protein Toll-like receptor 4 (TLR4) (Beutler and Poltorak, 2001). Different TLRs respond to different pathogen components thus ensuring the correct process to deactivate the pathogen is produced. With surface protein MD2, the TLR4 cytoplasmic cell signalling pathway moves through MyD88, TRAF6, to IRAK where the pathway bifurcates. One pathway produces $\text{I}\kappa\text{B}$ which produces the nucleus translocator, $\text{NF}\kappa\text{B}$. The other pathway goes through MAP3 kinase pathway to eventually produce $\text{NF}\kappa\text{B}$. There are also links to other pathways including JNK/p38 which leads to apoptosis (programmed cell death) through caspase

mediators (Beutler and Poltorak, 2001). NF κ B then activates the production of various messenger RNA proteins, stimulating the cell to produce other mediators. Intravenous LPS also activates capillary endothelium. It appears endothelial cells rapidly undergo apoptosis rather than necrosis from direct cell toxicity (Hack and Zeerleder, 2001).

The initial phase of intravenous LPS results in leukopaenia, reduced cardiac output and fall in arterial blood pressure. Pulmonary arterial blood pressure can increase and this is observed more commonly in species with PIMs (Parkhabar *et al.*, 2005), and is speculated to result from released thromboxane A₂ and platelet-activating factor (DeLong *et al.*, 1999). An improvement in these signs occurs 4-6 hours following mild-moderate doses of injectable LPS, although in clinical situations the production of LPS may be ongoing and the signs may worsen.

In presence of LPS, changes in lung compliance and hypoxaemia can be observed with increased alveolar-arterial oxygen partial pressure difference (decreasing PaO₂/FIO₂ ratio) from increased shunt and widened diffusion barrier. Neutrophil trapping occurs in the pulmonary capillaries with intravenous (IV) or intra-alveolar administration of LPS. Very few neutrophils migrate into the alveoli when LPS is given intravenously, compared to intra-alveolar route administration of LPS (Reutershan and Ley, 2004), (Kuebler, 2005).

1.3 Cytokines and chemokines

Pro-inflammatory cytokines include TNF α , IL-1 α , IL-1 β and IL-6. IL-1 α is released from macrophages, endothelial cells and fibroblasts, and IL-1 β is released from natural killer cells, macrophages and monocytes. IL-6 is released from fibroblasts, endothelial cells, neutrophils, monocytes and eosinophils and has both pro- and anti-inflammatory actions. IL-10 is an anti-inflammatory cytokine for inhibition of cytokine production in monocytes. TNF- α is produced by monocytes, macrophages, T and B cells, fibroblasts, neutrophils, natural killer cells and endothelial cells. It has both paracrine and endocrine functions promoting inflammation and immune system functions. Transforming growth factor beta (TGF- β) is released from macrophages, lymphocytes and dendritic cells.

TGF- β has immunoregulatory functions and is chemotactic for leukocytes, although inhibits them once they are activated (Rankin, 2004).

Chemokines are released to attract certain cells towards the site of injury. These are small proteins (8-10 kDa) and approximately 50 chemokines have so far been identified (Tizard, 2004). Nomenclature depends on spacing of cysteine residues, and IL-8 is now known as CXCL-8. IL-8 is produced by stimulated macrophages to attract and activate neutrophils. Monocyte chemotactic protein (MCP-1 or CCL-2) is produced by macrophages, T-cells, fibroblasts and endothelial cells to attract other monocytes to help with clearing debris and repair (Tizard, 2004).

1.4 Macrophages

Macrophages are part of the innate immune system (mononuclear phagocyte system) and act as sentinel cells, killing invading pathogens and play a role in triggering acquired immunity. Macrophages are specialized matured monocytes found in a number of tissues such as lymph nodes and bone marrow, serosa, connective tissue (histiocytes) and the spleen. Macrophages found in the circulation are discussed further below. Macrophages contain all the apparatus to manufacture and secrete cytokines once they have been activated (Tizard, 2004). Macrophages found in various organs differ in their function. Furthermore, macrophages found in the same organs also comprise a heterogeneous population. In the lung, macrophages are found in the airways, alveoli, interstitium and the capillaries (Brain, 1992)

1.4.1 Pulmonary Intravascular Macrophages (PIMs)

PIMs are firmly adhered to the endothelial cells of blood vessels within the lungs (Brain, 1992), (Longworth, 1997), (Staub, 1994), (Warner and Brain, 1990). These cells have the characteristics of mature tissue macrophages rather than monocytes, and form membrane adhesion complexes with endothelial cells (Longworth, 1997). PIMs are larger than monocytes (20-80 μ m) with more developed Golgi apparatus, rough endoplasmic reticulum and mitochondria. Their plasma membrane has a glycocalyx with micropinocytosis vermiformis indicating active endocytosis (Longworth, 1997). The

function of PIMs is rapid phagocytosis of invading bacteria, cellular debris, endotoxin and dying erythrocytes within the pulmonary circulation. Bacteria bind to the macrophage and stimulate the production of TNF- α and IL-1 which leads to activation of endothelium and expression of adhesion molecules leading to recruitment of neutrophils. The neutrophils then ingest the bacteria, undergo apoptosis, and are ingested by the macrophage thus preventing release of breakdown products into the circulation (Tizard, 2004). PIMs also promote recruitment of platelets in lung microvessels (Singh *et al.*, 2004). The sequestered platelets contain IL-8 and may promote recruitment of neutrophils.

Species in the order Artiodactyla naturally have PIMs and include pigs, deer, camelids, sheep, goats and cattle (Atwal *et al.*, 1989), (Rybicka *et al.*, 1974), (Staub, 1994), (Warner *et al.*, 1986), (Winkler *et al.*, 1985), (Winkler, 1988). Species in the orders Perissodactyla (horse, burro) and Cetaceans (whales, dolphins) also possess PIMs (Brain *et al.*, 1999), (Kawashima *et al.*, 2004), (Parbhakar *et al.*, 2005), (Staub, 1994). PIMs have also been identified in some strains of domestic cat (Molina and Brain, 2007), (Schneeberger-Keeley and Burger, 1970). Neonatal animals of PIM-positive species do not have PIMs at birth and recruit these cells within a few weeks (Horiguchi *et al.*, 1996), (Warner *et al.*, 1986). Species proven not to have PIMs can have the ability to recruit them with hepatic injury and include mice, rats, dogs and humans (Brain *et al.*, 1999), (Gill *et al.*, 2008), (Staub, 1994).

It is unknown why some species have PIMs and others do not. The pulmonary circulation receives the entire cardiac output and PIMs are well positioned to thoroughly remove particles and back up the liver intravascular macrophage system (Warner *et al.*, 1987), (Warner and Brain, 1990). The presence of PIMs does carry a price, and species with PIMs exhibit more severe acute lung injury (ALI) due to endotoxaemia, compared to species without PIMs (Chen *et al.*, 2003), (Gill *et al.*, 2008), (Parkhabar *et al.*, 2005). Depletion of PIMs prior to endotoxin exposure can lessen the degree of ALI (Parbhakar *et al.*, 2005), (Singh *et al.*, 2004), (Gill *et al.*, 2008), (Staub *et al.*, 2001).

Animal species with PIMs mount a more aggressive response to small doses of LPS in the $\mu\text{g/kg}$ range. In contrast, species without PIMs require doses of LPS in the mg/kg range. (Frevert and Warner, 1992). However, upon recruitment of PIMs, rats become

highly sensitive to minute amounts of LPS and depletion of PIMs with gadolinium chloride reverts them to their original physiological phenotype (Gill *et al.*, 2008). Rabbits appear to be intermediate in their response to LPS as doses of 100 µg/kg can be fatal (Ben-Shaul *et al.*, 1999).

1.4.2 Hepatic Küpffer Cells

Macrophages in contact with the systemic circulation are also found in the liver (Küpffer cells) and spleen of all species, and are responsible for similar actions as PIMs. Species with no PIMs rely heavily on Küpffer cells to clear blood of bacteria. Species with PIMs also have Küpffer cells, and the macrophage responsible for pathogen removal is probably more dependent on entry point of the invading pathogen, and which organ first receives the pathogen-laden blood (first pass effect) (Warner, 1996). Küpffer cells will also remove effete PIMs, or their released particles from the circulation (Molina and Brain, 2007). Küpffer cells are stimulated by LPS to produce pro-inflammatory cytokines (TNF α , IL-1, IL-6, IL-12), nitric oxide, reactive oxygen free radicals, and eicosanoid mediators. Normally, stimulation is at a very low level with normal amounts of bacterial translocation from the portal circulation. With high concentrations of LPS, a profound response is produced. Chemokines are also produced to attract neutrophils (Szabo *et al.*, 2002). The neutrophils can cause further liver injury through release of oxygen free radicals and proteases. Endothelial cells close to the Küpffer cells are also activated to release pro-inflammatory mediators and nitric oxide. These mediators contribute to changes in the microcirculation and refractory hypotension with ongoing LPS stimulation (Su, 2002).

1.5 The Rabbit as a Model for Acute Lung Injury

When using laboratory animal models it must be realized that different species have different mechanisms to deal with pathogen invasion. The rabbit has frequently been used to reflect human conditions including endotoxaemia, smoke inhalation, lung aspiration and positive pressure lung ventilation (Frevert *et al.*, 2000), (Jesmin *et al.*, 2004), (Altemeir *et al.*, 2004), (Bidani *et al.*, 1999). Some species have different Toll receptor pathogen/toxin recognition systems and the rabbit has 57% identity with human TLR4

receptors for LPS, compared with 48% observed with rat or mouse (Matute-Bello *et al.*, 2008). Rabbits also respond to LPS with similar intensity as humans, yet have more NO reactivity towards pathogens compared to humans. Because of the rabbit's larger size, multiple blood sampling is possible, and systemic blood pressure can be measured using conventional equipment (Matute-Bello *et al.*, 2008), (Poli-de-Figueiredo *et al.*, 2008). There are conflicting and confusing reports as to whether rabbits possess PIMs. There are reports which indicate that PIMs are rarely found in comparison to species within the order Artiodactyla (Brain *et al.*, 1999), (Horiguchi *et al.*, 1996). There are also reports indicating PIMs are possibly present in rabbit lungs, and are capable of producing severe ALI. One study revealed the presence of irregularly shaped phagosomes containing electron dense material which corresponded to viral antiserum staining (Carrasco, 1991). In the rabbit, primary clearance of *E. coli* infusion from the bloodstream mainly occurs within the liver and spleen, and clearance values rise in the lung from 20% to 90% by 5 hours post-infusion (Rogers, 1957). Another study found that primary clearance of LPS takes place in the liver, spleen and lungs, and the lungs contained the greatest concentration of *Salmonella minnesota* LPS five minutes post-infusion (Mathison and Ulevitch, 1979). The rabbit is frequently used as a model to study ALI and therefore the role of PIMs in the production of ALI needs to be addressed (Frevert *et al.*, 2000), (Matute-Bello *et al.*, 2008).

2 HYPOTHESIS

- a. Rabbits have pulmonary intravascular macrophages
- b. Eliminating pulmonary intravascular macrophages reduces lung inflammation with endotoxin challenge

3 OBJECTIVES

- a. To establish the presence of pulmonary intravascular macrophages in the rabbit
- b. To understand the role of intravascular macrophages in endotoxaemia

4. RATIONALE

Species containing PIMs are more prone to ALI when exposed to bacteria or endotoxin. It is known that ALI is reduced when PIMs have been previously removed using gadolinium chloride. There are many causes of ALI such as smoke inhalation, ventilator-induced injury, endotoxin or pathogen invasion, and ALI can occur as a secondary problem to sepsis. The high mortality associated with ALI in all species makes it a priority to advance our knowledge and management of ALI.

The rabbit is commonly used as a model to investigate ALI, but the presence of PIMs plays a strong role in the outcome. If it is found the rabbit has PIMs then this species could be used to reflect outcomes pertaining to horses. On the other hand, if the rabbit does not contain PIMs then its use as an appropriate model for humans is further validated.

5. MATERIALS AND METHODS

Table 5.1. Reagents and their Sources

Reagents	Source
Mouse anti-human antibody MAC387	AbD Serotec,
Mouse anti-rabbit macrophage RAM 11	Dako Laboratories
HRP-linked goat anti-mouse antibody	BD Pharmaceuticals
Sheep anti-human von Willibrand's factor antibody	The Binding Site, CA, USA
HRP-linked donkey anti-sheep antibody	The Binding Site, CA, USA
Goat anti-rabbit TNF polyclonal capture antibody	BD Pharmingen
Biotinylated mouse anti-rabbit TNF detection antibody	BD Pharmingen
Recombinant rabbit TNF- α (111.4 ng/mL)	BD Pharmingen
Protease inhibitor cocktail	Sigma Co.
Cetyltrimethylammonium chloride (CTAC)	Sigma Co.
3', 5, 5' - tetramethylbenzidine hydrochloride	Sigma Co.
Gadolinium Chloride. Made to 1.0 mg/mL by a compounding pharmacy, WCVI	Sigma Co.
Endotoxin (<i>E.coli</i> 0127:B8). Made to 1 mg/mL by compounding pharmacy, WCVI	Sigma Co.
Peroxidase colour development kit (immunostaining)	Vector Laboratories, USA
Methyl green	Vector Laboratories, USA
Prilocaine/Lidocaine cream (EMLA cream)	Astra Pharmaceuticals
0.9% intravenous saline	Abbott Laboratories
Pentobarbital Euthanasia Solution (240 mg/mL)	Bimeda-MTC Animal Health Inc.,

5.1 Animals

The study was approved by the University of Saskatchewan Protocol Review Committee (protocol numbers 20040083 and 20060058) and young, adult purpose-bred New Zealand White rabbits (Charles River Laboratories, Canada) were housed in an area with a 12 hour light/dark cycle, and environmental enrichment according to Canadian Council for Animal Care guidelines. Rabbits were housed in pairs or separated depending on temperament. They were acclimatized to handling, the treatment room and restraint device for 1 week before study. Twenty-seven rabbits were used for this study and were fed a laboratory chow with free access to water. Twelve rabbits were used in the first part of this study, and the remainder used in the second part.

5.2 Experimental Design

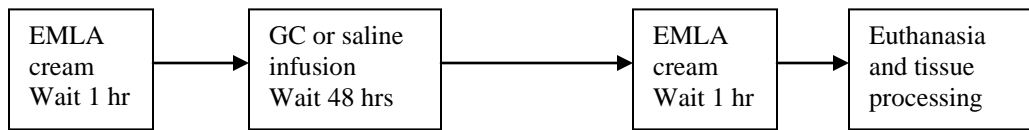
The study was divided into two parts (5.2.1 and 5.2.2). The first part of the study was to investigate the presence of PIMs in the lungs of normal rabbits, and after depletion using gadolinium chloride (GC) (Mizgerd *et al.*, 1996). Twelve rabbits (2.3 ± 0.1 kg bodyweight [mean \pm SD]) were used in the first part of the study, and divided equally into two groups. One group ($n=6$) received saline and another group ($n=6$) received GC, these groups are designated SAL and GC, respectively. Rabbits were randomly selected by paper draw to receive saline (SAL) or gadolinium chloride (GC).

5.2.1. Depletion of PIMs using GC

Rabbits were weighed and had prilocaine/lidocaine cream applied to the external surface of their ears one hour before placement of an over-the-needle catheter in a marginal ear vein (24G, Insyte Catheter, Becton-Dickinson and Co., Franklin Lakes, NJ, USA) and in the auricular artery (22G Insyte Catheter, Becton-Dickinson and Co., Franklin Lakes, NJ, USA). To deplete PIMs, six rabbits were administered GC (10 mg/kg intravenous [IV]). As a control, 0.9% saline (10 mL/kg IV) was administered to six other rabbits. Infusions were administered through the venous catheter during a 20 minute period using a computerized syringe pump (Medfusion 2100, Medexinc., Duluth, GA, USA). Once the infusion was finished, rabbits had all catheters removed and were returned to their cages.

Forty-seven hours later, prilocaine/lidocaine cream was again applied to the ears. An hour later, 5 mL of blood was withdrawn from the auricular artery, and divided between tubes containing EDTA, or no coagulant. Euthanasia was performed using an overdose of pentobarbital (200mg/kg IV) administered through a marginal ear vein. Tissues were collected as described later. The saline group (SAL) was considered to be the control group for both parts of the study (no treatment). Collection of data, tissues and samples is explained later in section 5.2.3 – 5.3.5.

Timeline for Part One: Removal of PIMs with Gadolinium Chloride



5.2.2 Endotoxin Administration

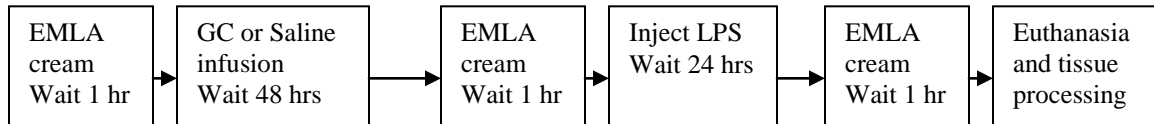
The second part of the study investigated the effect of removing PIMs on ALI after administration of LPS. Rabbits were pretreated with saline or GC before injection of LPS as described earlier, and groups designated SAL-LPS or GC-LPS, respectively. Rabbits were randomly divided into two groups according to paper draw for pretreatment and both groups received LPS injection.

A total of fifteen rabbits (2.2 ± 0.2 kg bodyweight) were used in total for this part of the study. The aim was to obtain six rabbits in each group. Four SAL-LPS rabbits died within 24 hours of administration of LPS and were replaced, one rabbit survived, leaving a final number of five rabbits in the SAL-LPS group. Six rabbits in the GC-LPS group survived to study completion. It was elected to not risk further loss of rabbits just to gain the final number of six rabbits in group SAL-LPS.

At 48 hours after administration of either saline or GC, and one hour after desensitizing the ear using local anaesthetic cream, each rabbit had an arterial blood sample anaerobically withdrawn for blood gas analysis of baseline values. Following this, each rabbit received a bolus intravenous injection of 100 µg/kg of LPS (*E.coli* 0127:B8) (Jesmin *et al.*, 2004). Rabbits were returned to their cages and frequently observed over the following 24 hours. At 24 hours post LPS injection, rabbits had their ears desensitized

and arterial blood samples withdrawn, followed by humane euthanasia with an overdose of pentobarbital using a marginal ear vein.

Timeline for Part Two: Removal of PIMs and Administration of Endotoxin



5.2.3 Systemic blood pressure measurement and blood sampling

Direct systemic arterial blood pressure was measured during infusion of saline or GC during the first part of the study. Saline filled non-compliant tubing connected the arterial catheter to a calibrated silicon-chip transducer (Truwave Disposable Pressure Transducer, Baxter Healthcare Corporation, Edwards Critical Care Division, Irvine, CA, USA) leveled to the sternum of the rabbit, and zeroed to atmospheric pressure. The transducer was connected to a physiological monitor (PB240 Operating Room Monitor, Puritan Bennet Corporation, Wilmington, MA, USA) displaying the systolic (SAP), mean (MAP) and diastolic (DAP) systemic arterial pressure. Arterial pressure measurements were recorded before infusion (baseline) and at five minute intervals during the 20 minute infusion period, and five minutes following termination of the infusion.

Arterial blood gas samples were anaerobically withdrawn from the arterial catheter, placed on ice, and analyzed within one hour using a blood gas analyzer (Ciba-Corning 238 blood gas analyzer, Ciba Corning Diagnostics, Halstead, Essex, UK). Blood electrolytes, glucose and lactate concentrations were also measured. Samples were withdrawn at baseline, after the infusion had finished, and 24 hours after LPS injection in the second part of the study.

Alveolar – arterial (A-a) oxygen partial pressure difference was calculated using archival recordings of barometric pressure from the Environment Canada website:

http://www.climate.weatheroffice.ec.gc.ca/climateData/hourlydata_e.html

The following equation was used:

$$PAO_2 = (\{\text{Barometric pressure} - 47\} \times 0.21) - (PaCO_2 / 0.8)$$

Where PAO_2 is partial pressure of O_2 in the alveolus (mmHg)

47 mmHg is saturated water vapour pressure at body temperature

0.8 is the standard respiratory quotient

0.21 represents the percentage of oxygen in room air, expressed as a decimal

5.3 Sampling and Processing

5.3.1. Collection of Tissues

All samples were stored on ice until further processed. After opening the thoracic cavity, the right lung was ligated at the hilus to avoid lavage fluid entering the lung. The pulmonary circulation was flushed using a 16G hypodermic needle inserted into the right ventricle with an incision made in the left atrium. Phosphate buffer solution (PBS) containing ethylenediaminetetraacetic acid (EDTA) was used as perfusion fluid. At least 100 mL was infused through the pulmonary circulation to remove erythrocytes and pulmonary monocytes. Following pulmonary circulation flush, a tracheotomy was performed distal to the larynx and a 6 Fr polyethylene feeding tube advanced to the level of the thoracic inlet to allow bronchoalveolar lavage (BAL). The tube was ligated with the trachea distal to the point of tracheal insertion. Lavage was performed three times by slowly infusing 10 mL PBS/EDTA through the feeding tube each time followed by aspiration. The first volume of BAL aspirate was kept separately from the last two flushes for future analyses. The caudal lobe of the right lung was removed, blotted of excess fluids and placed onto a pre-weighed tin foil dish. The remainder of the lung was placed into liquid nitrogen with the medial liver lobe and spleen. The frozen tissues were then placed into a -80°C freezer until required later for further analyses. The left lung was then perfused *in situ* with 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1M sodium cacodylate buffer through the feeding tube using a hydrostatic pressure of 25 cmH₂O, and

until fixative stopped flowing. The rabbit was suspended during lung infusion for 30 minutes and the left lung was removed after this period.

5.3.2. Lung and liver fixation and preparation

The left lung was removed and placed into 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1M sodium cacodylate buffer. The caudal lobe of the left lung was divided into seven sections and slices 2, 4 and 6 taken for further examination. Slices were placed into cassettes and left in 4% paraformaldehyde for 24 hours at 4°C. The tissues were then washed three times in PBS and left in PBS at 4°C until required. A portion of the right liver lobe was also taken, slices placed into cassettes and the tissues treated in the same manner. For immuno-electron microscopy, the cranial lung lobe and a portion of the right liver lobe was divided into 1 mm³ cubes and placed in 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1M sodium cacodylate buffer and after 3 hours, washed three times with 0.1M sodium cacodylate buffer, and left in this buffer solution at 4°C. Other 1mm³ cubes were used for regular ultrastructure electron microscopy. These tissue pieces were placed into 2% paraformaldehyde; 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for three hours, washed in three changes of 0.1M sodium cacodylate buffer and kept in this buffer at 4°C. For light microscopy, the tissue pieces in cassettes were processed through ascending concentrations of ethanol into paraffin using a tissue processor. Tissues were then blocked so that a section of each slice of lung was in one paraffin block. Sections 5µm thick were placed onto silane-coated slides and stained with haematoxylin/eosin or used for immuno-histochemistry.

5.3.3. Blood Samples

Blood smears were made using EDTA blood, air-dried, fixed with chilled 1% acetone for three minutes and then left overnight, after which they were stained with Wright's stain. Differentials were performed at a magnification of x100 by counting 100 cells using a crenallated pattern along the periphery and within the tail of the smear. A whole blood white cell count was performed after red cell lysis using a Neubauer haemocytometer and a commercial kit (Unopette Microcollection system, Becton Dickinson Vacutainer Systems, Becton Dickinson and Co., Franklin Lakes, NJ, USA). Coagulated blood

samples were left overnight at 4°C and centrifuged at 1400rpm for 15 minutes and the serum extracted. Serum was aliquoted into Eppendorf tubes and placed at -80°C.

5.3.4. Analysis of BAL Fluid

The fluid collected from BAL was centrifuged at 1500rpm for 10 minutes at 4°C and the supernatant from the first BAL flush aliquoted into Eppendorf tubes and placed at -80°C. The cell pellet was resuspended, and added to resuspended cell pellets from the remaining BAL flushes. Red cell lysis was performed by adding 9mL distilled water and 18 seconds later, adding 1mL 10X PBS. White cell count was performed using a haemocytometer. The tubes were centrifuged once again and the supernatant discarded. The cell pellet was resuspended and diluted to the appropriate amount using PBS/EDTA solution. Cytospin was performed using 100 µL (10^5 cells/slide) of the solution at 1800 rpm for five minutes. Slides were left to air-dry and were then fixed in chilled 1% acetone for three minutes, left overnight and then stained using Wright's stain. Differentials were performed at a magnification of x100 by counting the cells within ten random fields.

5.3.5 Wet/Dry Lung Ratio

Lung pieces were weighed in foil and placed in an incubator at 68°C for seven days after which time the foil and lung was reweighed. The ratio was calculated by dividing the wet weight of the lung piece by the dry weight. The difference in weight was assumed to be water loss.

5.4. Light Microscopy

5.4.1. Haematoxylin/Eosin Stain

Lung and liver tissue sections were stained using haematoxylin and eosin in a standard manner. Septal and alveolar heterophil (neutrophil) cells were counted in ten randomly selected fields at x100 power using light microscopy in lung tissue. Liver tissue was examined for infiltration of heterophils and a descriptive assessment made.

5.4.2. Immunohistochemistry

Slides of lung tissue were de-paraffinized with xylene and descending concentrations of ethanol. Endogenous hydrogen peroxide was removed using 0.5% hydrogen peroxide in methanol and antigen released using pepsin (2 mg/mL in 0.01N hydrogen chloride) at 37°C. Slides were blocked using bovine serum albumin (1% BSA in PBS). Primary mouse anti-human antibody MAC-387 (1:25 dilution in BSA) (Flavell *et al.*, 1987) and monoclonal mouse anti-rabbit macrophage RAM-11 (1:50 dilution in BSA) were used to identify pulmonary macrophages on separate sections of lung (100 µL/section) (Welt *et al.*, 2000). After washing in PBS, secondary horse-radish peroxidase (HRP)-linked goat anti-mouse antibody (1:100 dilution in BSA) was applied for 30 minutes and then washed using PBS. Colour development was achieved using a commercial kit. Controls consisted of the same protocol except one section did not receive primary antibody, and another section received sheep anti-human von Willibrand's factor antibody (1:250 dilution in BSA) with secondary antibody specific for this antibody (HRP donkey anti-sheep antibody; 1:150 dilution in BSA) (Kockx *et al.*, 1993). Counter-staining was accomplished using methyl green.

5.5 Immuno-Electron Microscopy

Immuno-electron microscopy (IEM) was performed on tissues processed through several ascending concentrations of ethanol and into LR White resin (EM Sciences, USA) and resin hardened at -10°C with ultra-violet light for 48 hours. One micron sections were prepared and stained with toluidine blue to select appropriate areas for ultra-microtomy. Ultra-thin (0.1 micron) sections were cut from the tissue blocks and placed on nickel grids.

Nickel grids with sections were floated on a blocking buffer (1% BSA and 0.1% Tween-20 in tris-buffered saline; pH 7.9) for 30 minutes followed by incubation with rabbit TNF- α antibodies for one hour. Tissue sections were rinsed three times for five minutes each in Tris-buffered saline and incubated with appropriate gold conjugated secondary antibodies (1:100) for one hour followed by staining with 2% aqueous uranyl acetate and then lead citrate.

5.6 Myeloperoxidase Assay

Samples of liver and lung were homogenized in 50 mM HEPES buffer solution at a pH of 8.0 at a ratio of 200 mg tissue to 0.5 mL solution. Samples were centrifuged at 3,000 rpm for 20 minutes at 4°C and the supernatant discarded. The pellet was resuspended in 0.5% cetyltrimethylammonium chloride to the original volume. The sample was rehomogenized and centrifuged as before. The supernatant was removed for further analysis using a peroxidase colour substrate (TMB) and 1M sulphuric acid as stop solution. Samples of appropriate dilution using phosphate citrate buffer were placed in a standard 96 well plate. Baseline optical density was taken from wells with stop solution added before sample and TMB (time 0). Colour was allowed to develop in other wells for 2 minutes before stop solution was added. All chemicals were used at room temperature. The optical density was measured using an optical reader at a wavelength of 450 nm. The density of the control (time 0) was subtracted from the sample reading (time 2 min), and this value used in statistical analysis (Schneider and Issekutz, 1996).

5.7 Enzyme Linked Immunosorbant Assay (ELISA)

ELISA was used to measure TNF- α protein concentrations in serum, BAL fluid, lung and liver tissue homogenate. Lung and liver tissues were homogenized in HBSS diluted with PBS solution containing protease inhibitor cocktail at a ratio of 100 μ L cocktail to 10mL HBSS solution. Ratio of tissue to solution was 100 mg:1mL solution. Homogenates were centrifuged for 20 minutes at 3,000 rpm at 4°C. The supernatant was collected and stored at -80°C until further analysis. Preliminary ELISA was performed to establish optimum concentrations of capture and detection antibodies. Capture antibodies were studied at a range 5 – 12.5 μ g/mL (standard rabbit recombinant TNF α 0.5 ng/mL; detection antibody 2.5 μ g/mL). Detection antibody was tested at a range of 0.125 – 5.0 μ g/mL (capture antibody 10 μ g/mL; standard rabbit recombinant TNF α 0.5 ng/mL). Based on results, 10 μ g/mL capture antibody with 5 μ g/mL detection antibody were selected for use in ELISA plates testing samples.

For ELISA of samples, 96-well plates were coated with 50 μ L capture antibody (10 μ g/mL goat anti-rabbit TNF polyclonal antibody) diluted with sodium phosphate buffer, and incubated at 4°C overnight. Next day, excess capture antibody was removed and 100 μ L blocking buffer (1% BSA in PBS) was added to each well and incubated at room temperature for two hours. Blocking buffer was removed and wells were washed with PBST (PBS containing 0.05% Tween-20). Standard dilutions of recombinant rabbit TNF- α (111.4 ng/mL; BD Pharmingen) at a range of 0.134 – 22 ng/mL in blocking buffer–Tween were made. Standards and samples (100 μ L of each) were added to the wells and incubated at 4°C overnight. After removing excess standards and samples, wells were washed with PBST and 100 μ L of detection antibody (biotinylated mouse anti-rabbit TNF monoclonal antibody) were added to each well after dilution with blocking buffer–Tween. The plate was left at room temperature for 90 minutes. Excess detection antibody was removed and wells were thoroughly washed. Streptavidin-Horseradish peroxidase (100 μ L 1:5000 in 1% BSA/PBS) was added to each well and incubated for 90 minutes at room temperature. Wells were thoroughly washed with PBST and 100 μ L TMB substrate (Substrate Reagent Pack, R&D Systems, Minneapolis, MN, USA) was added to each well, and incubated in the dark at room temperature for 30 minutes. The reaction was stopped at this time by adding 100 μ L 2N sulphuric acid (Stop Solution, R&D Systems, Minneapolis, MN, USA) to each well. Optical density was measured using a microplate reader at a wavelength of 450 nm. Standard curves were created to calculate the concentration of TNF- α in sample wells. No cross-reactivity with other cytokines and 10% cross-reactivity with mouse and human TNF are reported. The lower limit of detection was 0.18 ng/mL.

Due to difficulties in obtaining commercial reagents for examination of rabbit tissues and fluids, another laboratory ran their established ELISA protocols for rabbits on BAL fluid, serum, and lung and liver tissue for MCP-1 (CCL-2) and IL-8 (CXCL-8) chemokines (Kajikawa *et al.*, 1996). Lower limit of detection was 0.1 pg/mL for IL-8 and 0.16 ng/mL for MCP-1.

5.8 RT-PCR

Total RNA was isolated from lung tissues by sequential extraction with TRIzol reagent (Invitrogen) followed by treatment with RNase-free DNase I (Qiagen) and purification by RNeasy mini-columns (Qiagen) according to manufacturer's instructions. RNA was quantified by spectrophotometric analysis.

Superscript III one-step RT-PCR system with platinum *Taq* DNA polymerase (Invitrogen) was used for semi-quantitative analysis of the levels of expression of TNF- α , IL-1 β and IL-6 mRNA in lung and liver tissues of variously treated rabbits. Reactions were performed as directed by the manufacturer, using the oligonucleotide pairs in Table 5.8.

Each reaction was performed using 11.5ng/ μ L of total RNA and the thermocycler was programmed for reverse-transcriptase at 55°C for three minutes, initial denaturation of the cDNA at 94°C for two minutes, 30 amplification cycles, each of which consisted of 94°C for 15 seconds, 59°C for 30 seconds, and 68°C for one minute followed by a final extension at 68°C for five minutes. To ensure lack of DNA contamination, two units of platinum *Taq* DNA polymerase was substituted for the Superscript III/RT/Platinum *Taq* mixture in the reaction. A negative control reaction consisted of all of the components of the reaction mixture except RNA. Amplified PCR products were electrophoresed on a 1% TAE-agarose gel, stained with ethidium bromide and photographed under UV light.

Table 5.8: Primer Sequences for Rabbit

Gene	Forward Primer	Reverse Primer
TNF α	5'-ATGGTCACCCTCAGATCA-3'	5'-CTGAAGAGAACCTGGGAG-3'
IL-1 β	5'-ATGGCAACAGTACCTGAG-3'	5'-CGACTGACAAGACCTGCC-3'
IL-6	5'-ATGAACTCCTTCACAAGC-3'	5'-CAGCTCCTTGATGGTCTC-3'
GAPDH	5'GGCAAAGTGGATGTTGTCGCC-3'	5'GGGCACCAGCATCACCCCACT-3'

5.9 Statistical Analysis

Data were assessed for Gaussian distribution by graphing and visual examination.

Data from individual MPO and TNF α ELISA plates (3 tests) were analyzed using Kruskal-Wallis 1-way ANOVA followed by Dunn's test. Once it was established that each of three MPO or TNF α ELISA plate tests were not different from each other and had normal distribution using graphing techniques for a particular fluid or tissue sample, data were pooled and re-analyzed using 1-way ANOVA followed by Bonferroni post-hoc test.

A 2-way ANOVA for repeated measures with Bonferroni post-hoc test was used for systemic blood pressure, and saline or gadolinium chloride pre-infusion and post-infusion electrolytes, glucose and lactate, with time and treatment as factors. Wet/dry weight was examined using 1-way ANOVA with Bonferroni's post-hoc test.

White blood cell counts and differentials, slide cell counts, blood gases and pH, electrolytes, glucose, lactate, anion gap, A-a O₂ difference, septal RAM-11 count, RT-PCR data, serum TNF α , and IL-8 and MCP-1 data were compared using a Kruskal-Wallis 1-way ANOVA followed by Dunn's test, or a Mann-Whitney test for nonparametric data.

A Chi-Square test was used to test whether pretreatment with gadolinium chloride had an influence on survival when given before LPS compared to saline pretreatment.

All graphs and calculations were performed using computer software (GraphPad Prism 4.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Significance was taken when $P < 0.05$.

6 RESULTS

6.1 Search for PIMs

The following are the results from this study.

- a. There were no differences between the saline and the GC groups in the mean arterial blood pressure during infusion of saline and GC (Table 6.1.1)
- b. Arterial blood gases (Table 6.2.2), electrolytes, blood glucose and blood lactate concentrations (Table 6.1.1) were not different between SAL and GC. Potassium concentration was significantly lower in the SAL group after the saline infusion compared to before infusion, but remained within normal physiological limits.
- c. There were no differences in the total cell counts in the BAL fluid obtained from the SAL and the GC groups. All cells found were alveolar macrophages in both groups (Table 6.1.2).
- d. Lung sections stained with H&E showed normal lung architecture in samples obtained from both SAL and GC rabbits (Figure 6.1.1).
- e. Light microscopy on epoxy-embedded toluidine blue stained sections revealed macrophages in alveolar septum of lungs from both SAL and GC rabbits. In the lung sections from GC-treated rabbits, many septal cells with condensed nuclei were observed, as an indication of apoptosis (Figure 6.1.2:A and B).
- f. Routine electron microscopy revealed PIMs in the septal capillaries with characteristic close adherence to the endothelium, many lysosomes, and slightly indented nucleus (Figure 6.1.3: A, B and C).
- g. Immunohistology performed with anti-macrophage antibodies (MAC-387 and RAM-11) stained cells in the alveolar septa and within the alveoli (Figure 6.1.4: RAM-11: A and B).

- h. The numerical counts of RAM-11 positive septal cells did not show any differences between the two groups ($P=0.2$; Figure 6.1.5).
- i. Lung tissue myeloperoxidase activity was not different between SAL and GC groups (Figure 6.2.5).

6.2 Effects of Endotoxin (Part Two)

6.2.1 Mortality

Rabbits became visibly lethargic by two hours after injection of LPS. After injection of LPS, one out of six rabbits survived to 24 hours after LPS injection in group SAL-LPS and four of the five dead rabbits were replaced (SAL-LPS: $n = 5$) (Figure 6.2.1). In group GC-LPS, six out of six rabbits survived to 24 hours after LPS injection ($n = 6$). Chi-square test found that rabbits receiving saline pretreatment were 3.5 times more likely to die, but $P=0.063$ and the curves were not significantly different. Withdrawal of blood was difficult in all rabbits after they had received LPS, compared to otherwise healthy rabbits and accounts for missing data in the LPS groups.

6.2.2 Arterial Blood Gas Analysis, Electrolytes and Acid/Base Balance

Blood gas sample data from groups SAL and GC from Part One of this study were used as controls to compare data from groups SAL-LPS and GC-LPS. Due to difficulties in obtaining blood samples from LPS treated rabbits, non-parametric statistics were used and data expressed as median (range) (Table 6.2.2).

There was no differences in PaO_2 , Alveolar-arterial difference, pH, lactate, anion gap, sodium, chloride, ionised calcium and potassium ion concentrations, and base excess/deficit in GC-LPS or SAL-LPS groups compared to SAL or GC rabbits, or with each other.

Bicarbonate ion concentrations and PaCO_2 were significantly lower in GC-LPS group rabbits compared to group SAL (Table 6.2.2).

6.2.3 Cell Counts and Histology

The total blood leukocyte count was significantly lower in rabbits belonging to the SAL-LPS group compared to the SAL control group from Part One of this study. There was no difference in white cell counts between other groups, or in the white blood cell differential (Table 6.2.3).

There were no significant differences in total leukocyte counts in BAL fluid using a haemocytometer, or in macrophage numbers on cytopsin, between any groups. With cytopsin, one heterophil was observed in one rabbit from each SAL-LPS and GC-LPS groups (Table 6.1.2).

There was no difference in number of septal heterophils between SAL-LPS and GC-LPS groups, and both LPS treated groups had greater numbers of septal heterophils compared to their respective control groups (Figure 6.2.3.1 and Figure 6.2.3.2: A, B and C).

In H&E stained liver tissue there were a small number of heterophils infiltrating liver tissue in three GC-LPS rabbits. In one rabbit from the SAL-LPS group the liver appeared hyperaemic with many heterophils (Figure 6.2.3.3: A and B).

6.2.4 Wet/Dry Ratio of Lung Tissue

There was no significant difference in water content of lung tissue between any groups (Table 6.2.4).

6.2.5 Myeloperoxidase Assay

It was established that data from three separate analyses did not differ therefore data from each analysis were pooled (Figure 6.2.5). Myeloperoxidase activity was significantly higher in lung tissue of rabbits in group SAL-LPS compared to group GC-LPS ($P < 0.05$).

6.2.6 ELISA for TNF α

BAL fluid, liver and lung homogenate were tested three times using duplicate wells in each plate, and the data pooled for analysis once it was established each plate did not

differ from each other. Serum was tested twice as there was limited amount of material and nonparametric statistics used. Standard curves were of good quality on all plates. TNF α data is presented as mean \pm SD. IL-8 and MCP-1 data were obtained from another laboratory using one test run (Kajikawa *et al.*, 1996).

6.2.6.1 Bronchoalveolar lavage (BAL) fluid TNF α , IL-8 and MCP-1 concentration

There was a significantly higher concentration of TNF α in BAL fluid of rabbits in SAL control group compared to SAL-LPS and GC-LPS (Figure 6.2.6.1: A). Rabbits in the GC-LPS group had significantly lower TNF α concentrations compared to GC and SAL control rabbits. There were no differences between all treatment groups with IL-8 and MCP-1 concentration (Figure 6.2.6.1: B and C).

6.2.6.2 Serum TNF α , IL-8 and MCP-1 concentration

TNF α concentrations were found to be significantly higher in the serum of SAL-LPS rabbits compared to rabbits in group GC-LPS, GC and to SAL control (Figure 6.2.6.2: A). MCP-1 and IL-8 concentrations were not found to be significantly different between groups (Figure 6.2.6.2: B and C).

6.2.6.3 Lung and Liver Tissue TNF α , IL-8 and MCP-1 concentration

There were no significant differences in IL-8 concentrations in lungs or liver of rabbits between all groups (Figure 6.2.6.3: A and B). There were no significantly different MCP-1 concentrations between all groups in lung or liver tissue (Figure 6.2.6.3: C and D). There were no significant differences in TNF α concentrations between all groups in lung and liver tissues (Figure 6.2.6.3: E and F).

6.2.6.4 Immuno-electron microscopy for TNF α

Immuno-electron microscopy revealed labeling for TNF α in PIMs of control and LPS rabbits. There appeared to be more gold particles observed in PIMs of LPS-treated rabbits (Figure 6.2.6.4: A and B).

6.2.7 RT-PCR

6.2.7.1 Lung tissue TNF- α , IL-1 β and IL-6 mRNA

There was a significantly higher fold increase in TNF α (Figure 6.2.7.1), IL-1 β (Figure 6.2.7.2) and IL-6 mRNA (Figure 6.2.7.3) in lung tissue of SAL-LPS rabbits compared to GC-LPS rabbit lungs. There was a significant fold increase in IL-1 β and IL-6 mRNA in SAL-LPS compared to SAL control rabbits.

Haemotoxylin and Eosin Staining and Immunohistology of Rabbit Lung Tissue:

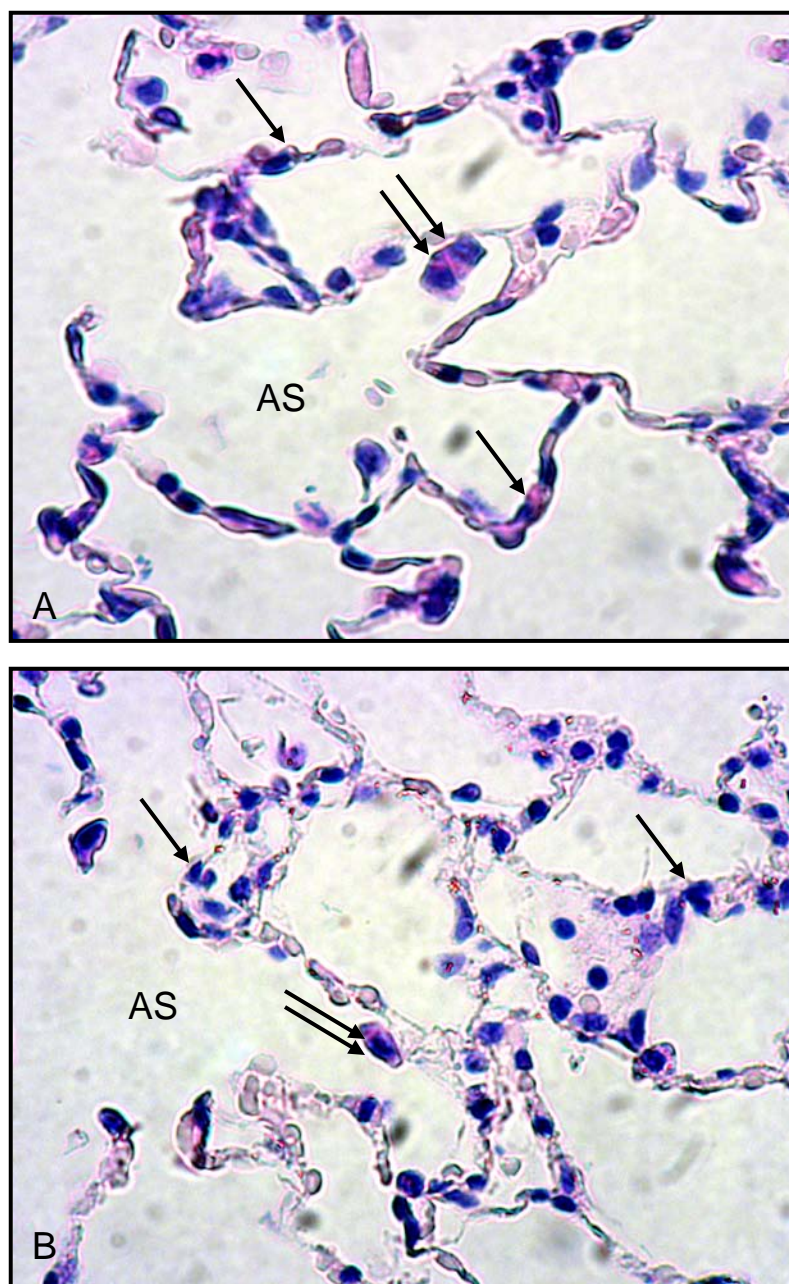


Figure 6.1.1: H&E staining of lung tissue from saline (A) and GC (B) pre-treated rabbit. AS represents Alveolar Space. Single arrows show Alveolar Septae, double arrow: alveolar macrophage. Original magnification: 10 (A) and 40 (B).

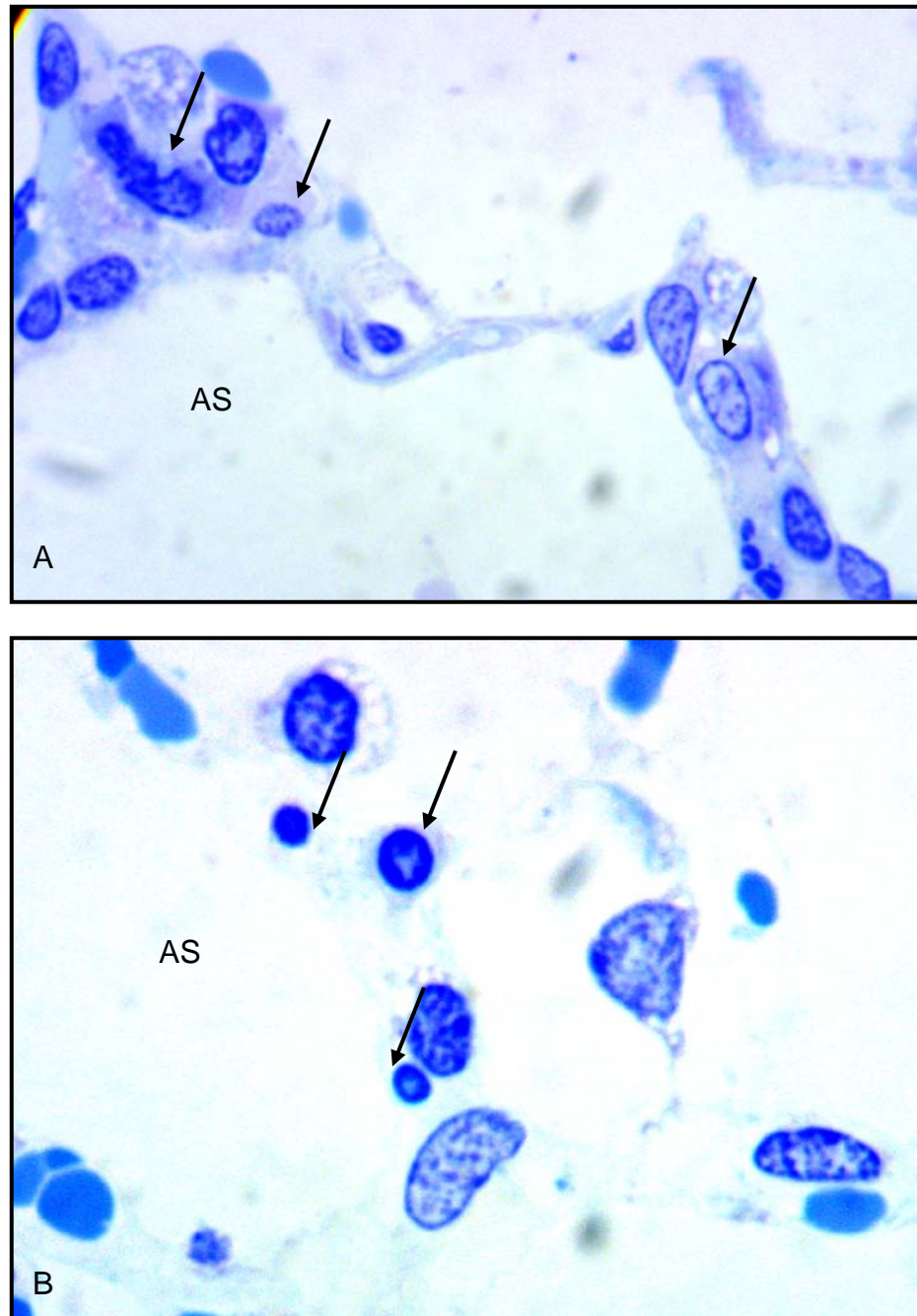


Figure 6.1.2: Lung sections from a saline-treated (A) and gadolinium-treated rabbit (B) show healthy (A) and apoptotic (B) pulmonary intravascular macrophages (single arrows). AS: Alveolar Space.

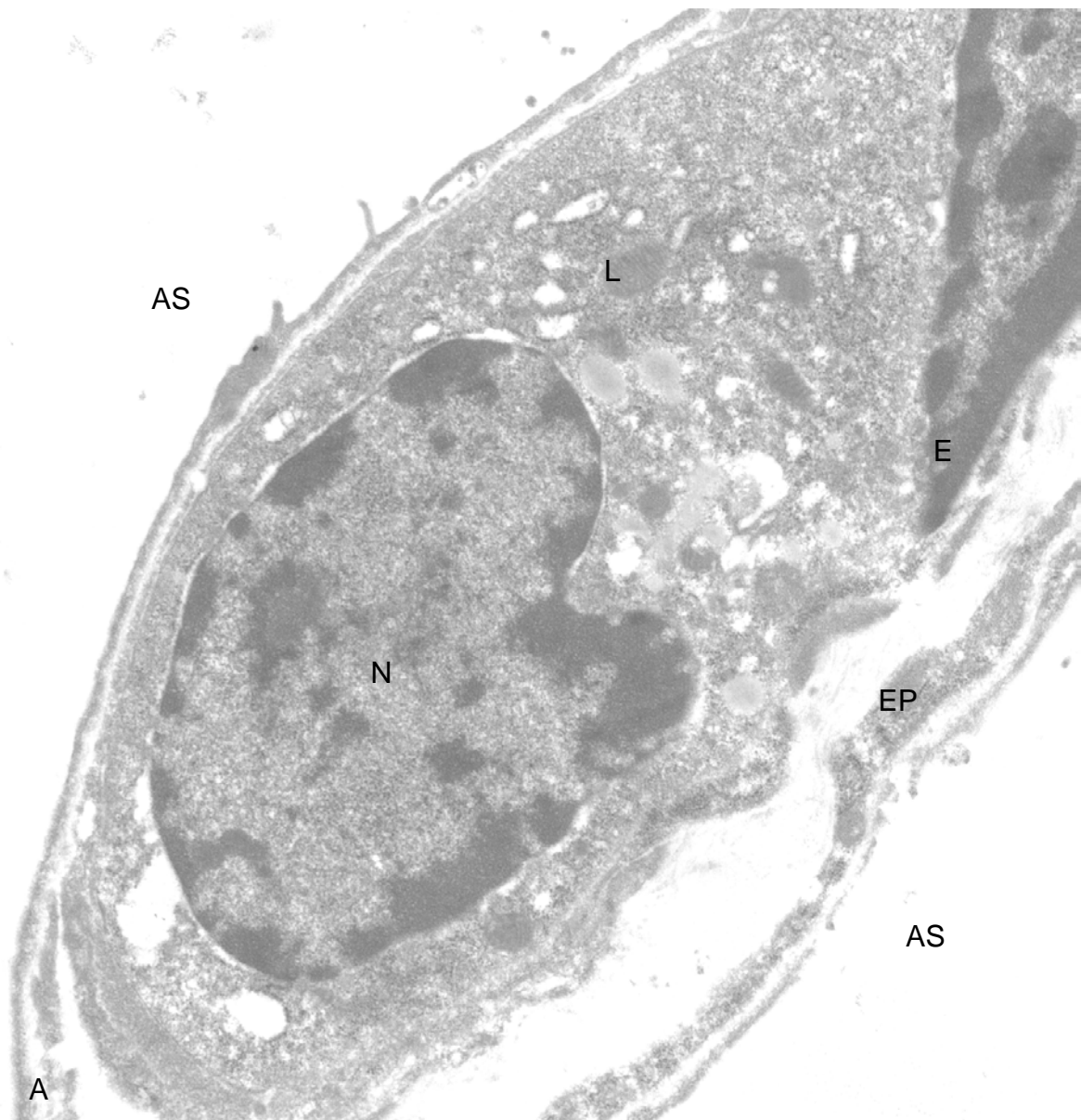


Figure 6.1.3: Electron micrograph of a pulmonary intravascular macrophage (PIM) from septal lung capillaries of a rabbit from group SAL. The PIM contains a nucleus (N), and many lysosomes (L) and is attached to the capillary endothelium (E). EP: Epithelium; AS: Alveolar space. Original magification: 10,000

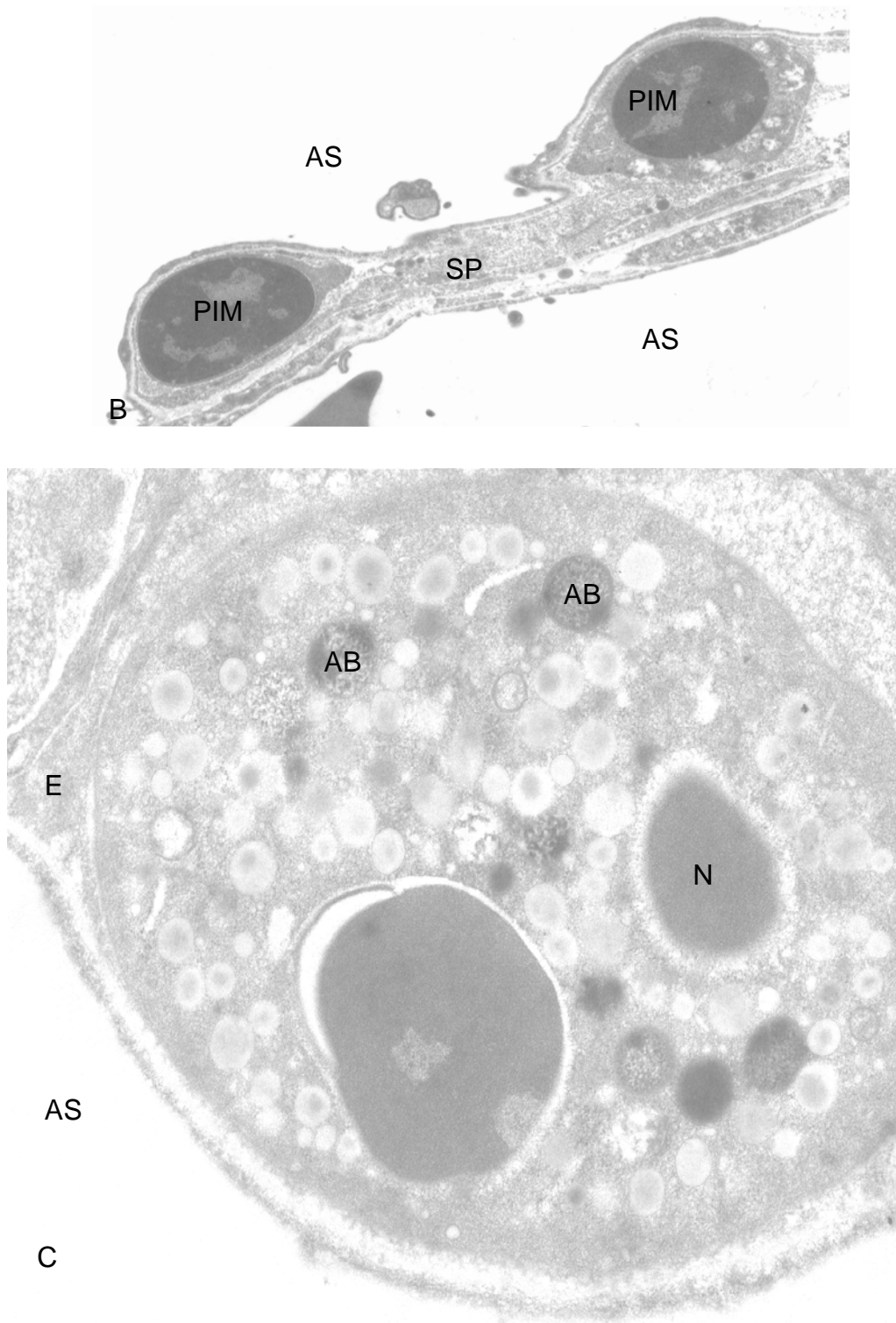


Figure 6.1.3: Figure B shows two apoptotic PIMs and C shows another apoptotic PIM with condensed nucleus (N) and apoptotic bodies (AB). Many vacuoles with digested material are also seen. E: Endothelium; SP: Alveolar Septum; AS: Alveolar Space. Original magnification: B: 4,500; C: 12,000.

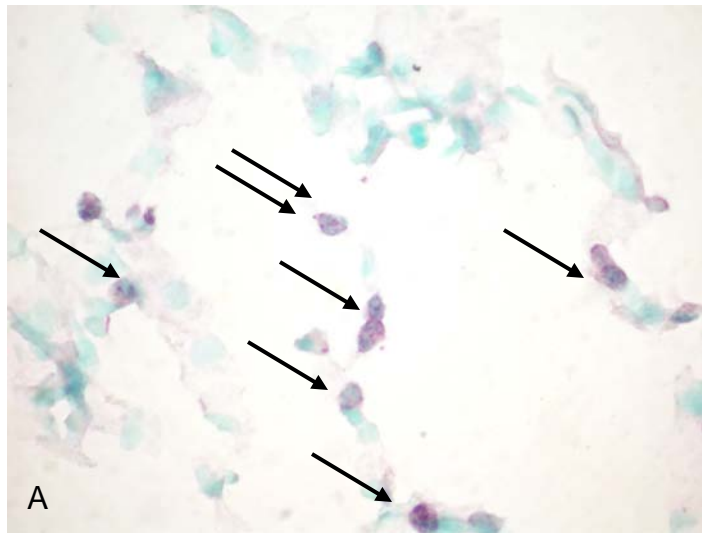


Figure 6.1.4: Immunostaining with anti-macrophage antibody RAM-11 shows staining in alveolar macrophages (double arrows) and septal macrophages (single arrows). E: Endothelium. Original magnification: A: 40; B:100

Graph of RAM-11 septal macrophage count:

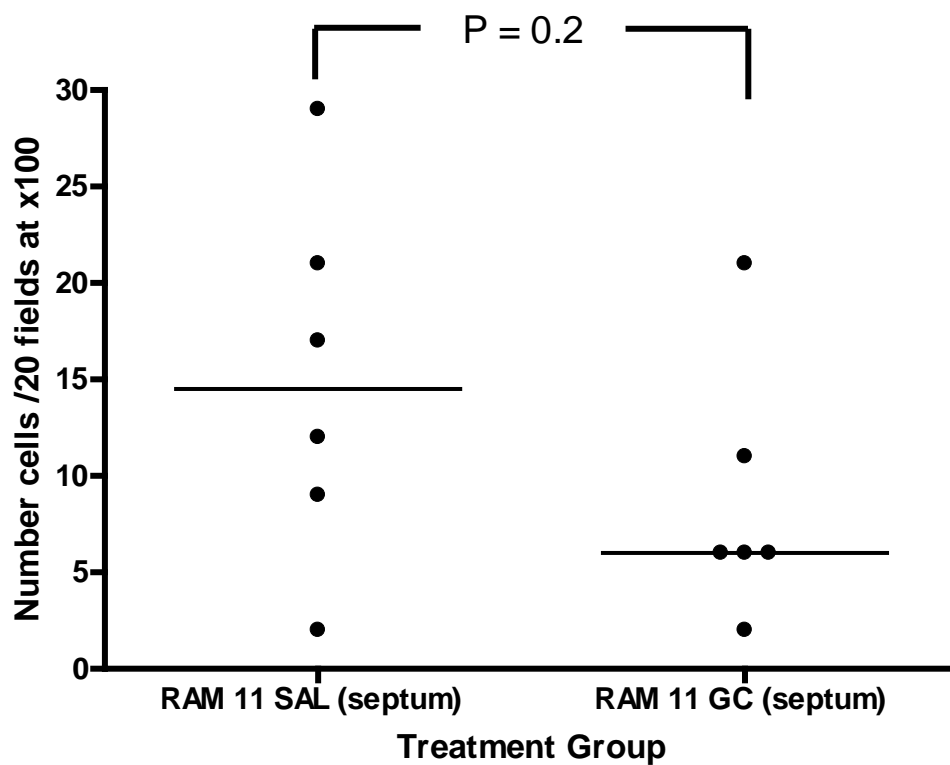


Figure 6.1.5: Graph of RAM-11 positive cell count in the alveolar septum of SAL and GC treated groups. Bars represent median values.

Table 6.1.1 of Mean Arterial Blood Pressure, Lactate, Glucose, Potassium and Ionized Calcium before, during and after Infusions (Part One) (Mean \pm SD; n=6):

Time (minutes)	Mean Arterial Blood Pressure (mmHg)		Glucose (mmol/L)		Lactate (mmol/L)		Electrolytes (mmol/L)			
							Potassium		Ionized Calcium	
	SAL	GC	SAL	GC	SAL	GC	SAL	GC	SAL	GC
Baseline (time 0)	80 \pm 9	80 \pm 7	8.2 \pm 0.7	9.0 \pm 1.9	2.8 \pm 1.5	4.2 \pm 2.1	3.87 \pm 0.11 ^a	3.77 \pm 0.29	1.67 \pm 0.06	1.54 \pm 0.10
5	75 \pm 10	78 \pm 6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
10	76 \pm 7	74 \pm 7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
15	72 \pm 13	74 \pm 7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
20	72 \pm 11	72 \pm 7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5 minutes after end of infusion	74 \pm 11	76 \pm 7	8.6 \pm 0.9	8.6 \pm 1.8	2.0 \pm 0.7	2.8 \pm 1.3	3.30 \pm 0.29 ^a	3.45 \pm 0.44	1.54 \pm 0.07	1.46 \pm 0.10

n/a: Not available. Use of the same letter indicates significant difference from each other for that variable (P<0.05)

Table 6.1.2 of Cell counts from Bronchoalveolar Lavage (BAL) (Mean \pm SD):

	Before LPS Injection Part One Study		After LPS Injection Part Two Study	
	Saline (n=6)	GC (n=6)	Saline (n=5)	GC (n=6)
BAL white cell count (10 ⁵ /mL)	11.5 \pm 6.5	15.4 \pm 7.3	8.9 \pm 4.1	11.1 \pm 5.1
Macrophage: Heterophil number (From 10 fields at x100)	100:0	100:0	99:1	99:1

Table 6.2.2 of Arterial Blood Gases, Electrolytes and Acid Base (Median[range]):

Variable Median (range)	Pretreatment (Part One Study)		24 hours after LPS injection	
	SAL (n=6)	GC (n=6)	SAL-LPS (n=3)	GC-LPS (n=4)
PaO ₂ (mmHg)	79 (66-131)	82 (71-89)	114 (65-121)	93 (85-116)
Alveolar-arterial O ₂ Difference (mmHg)	22 (0-35)	22 (18-37)	23 (14-52)	32 (23-42)
PaCO ₂ (mmHg)	38 (34-41) ^a	36 (34-39)	25 (23-37)	27 (18-30) ^a
Bicarbonate (mmol/L)	26 (24-31) ^a	25 (20-28)	17 (16-23)	18 (13-19) ^a
pH (Units)	7.46 (7.43-7.50)	7.46 (7.36-7.49)	7.44 (7.43-7.50)	7.46 (7.43-7.48)
Base Excess/Deficit (Units)	2.3 (-0.2-7.0)	1.7 (-5.3-4.6)	-3.9 (-5.8-0.0)	-3.9 (-7.1- -2.4)
Sodium (mmol/L)	137 (131-139)	138 (136-140)	141 (136-142)	140 (136-142)
Chloride (mmol/L)	102 (99-104)	101 (99-103)	107 (102-113)	110 (98-112)
Potassium (mmol/L)	3.8 (3.7-4.0)	3.7 (3.3-4.1)	3.7 (3.7-4.7)	3.8 (3.5-4.6)
Ionised Calcium (mmol/L)	1.6 (1.5-1.6)	1.5 (1.4-1.7)	1.3 (1.1-1.5)	1.3 (1.2-1.5)
Glucose (mmol/L)	8.1 (6.8-9.1)	8.4 (6.7-13.2)	7.1 (6.9-7.6)	6.3 (5.5-7.4)
Lactate (mmol/L)	2.0 (1.6-5.5)	4.3 (1.1-7.9)	3.5 (2.8-4.0)	3.3 (2.2-6.2)
Anion Gap (mmol/L)	12.7 (4.9-17.2)	15.7 (13.4-21.7)	16.2 (15.5-20.2)	17.8 (15.1-22.2)

Unpaired samples. Use of the same letter indicates significant difference from each other for that variable (P<0.05). Some data in LPS groups missing due to difficulty obtaining blood samples.

Table 6.2.3 of Blood White Cell Count and Differential (Mean \pm SD):

	Normal Rabbit*	Before LPS Injection Part One Study		After LPS Injection Part Two Study	
Differential		SAL (n=6)	GC (n=6)	SAL-LPS (n=3)	GC-LPS (n=5)
Blood WBC ($\times 10^9/L$)	5 - 12	6.7 ± 2.1	7.9 ± 1.0	5.0 ± 2.9	5.8 ± 3.5
Blood Heterophil (%)	30 - 50	12.0 ± 7.4	20.0 ± 8.5	19.7 ± 8.5	18.8 ± 9.1
Blood lymphocytes (%)	30 - 60	78.0 ± 6.0	67.7 ± 8.0	73.0 ± 9.9	71.5 ± 7.0
Blood Monocytes (%)	2 - 10	8.6 ± 1.7	10.3 ± 7.8	6.3 ± 5.5	8.0 ± 5.6

Unpaired samples. Some data in LPS groups missing due to difficulty obtaining blood samples.

* <http://www.vet.uga.edu/vpp/clerk/lester/index.php>. Accessed April 2009

Table 6.2.4 of Lung Wet/Dry Ratios (Mean \pm SD; n=6)

Group SAL	Group GC	Group SAL-LPS	Group GC-LPS
5.4 ± 0.2	5.3 ± 0.2	5.2 ± 0.4	5.4 ± 1.0

Survival Curves:

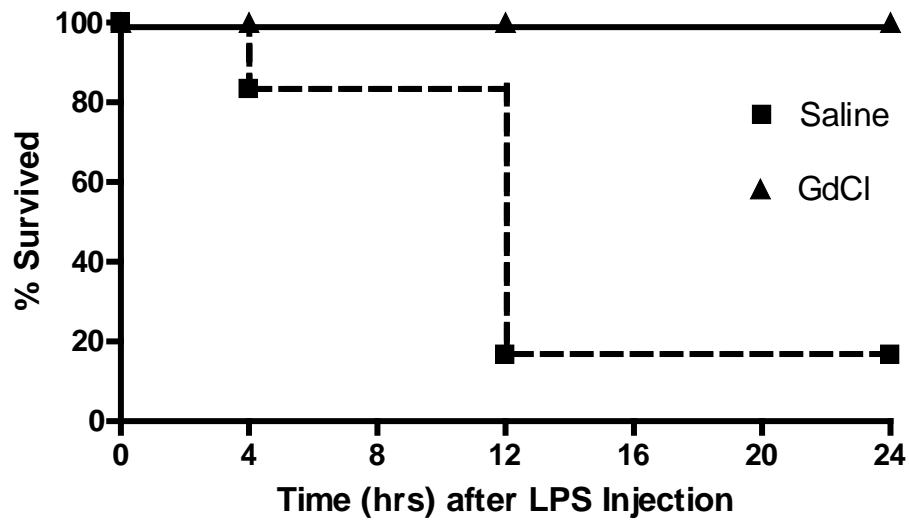


Figure 6.2.1: Survival Curve after administration of LPS to rabbits either pretreated with saline or gadolinium chloride. Mortality recorded at timepoints 4, 12 and 24 hours after LPS injection. Rabbits receiving saline were 3.5 times more likely to die, but curves are not significantly different: $P=0.063$.

Septal Heterophil Counts:

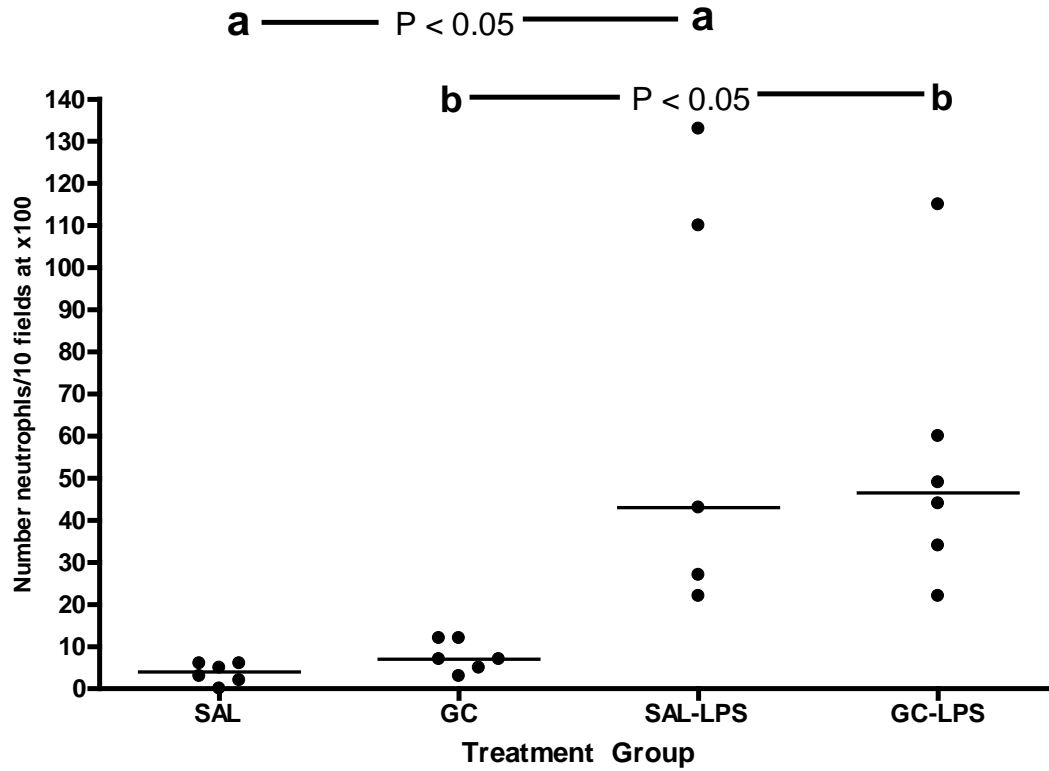


Figure 6.2.3.1: Comparison of septal heterophil numbers in rabbits pretreated with either saline or GC and then administered LPS. Bar represents median value.

Same letter indicates significant difference between groups.

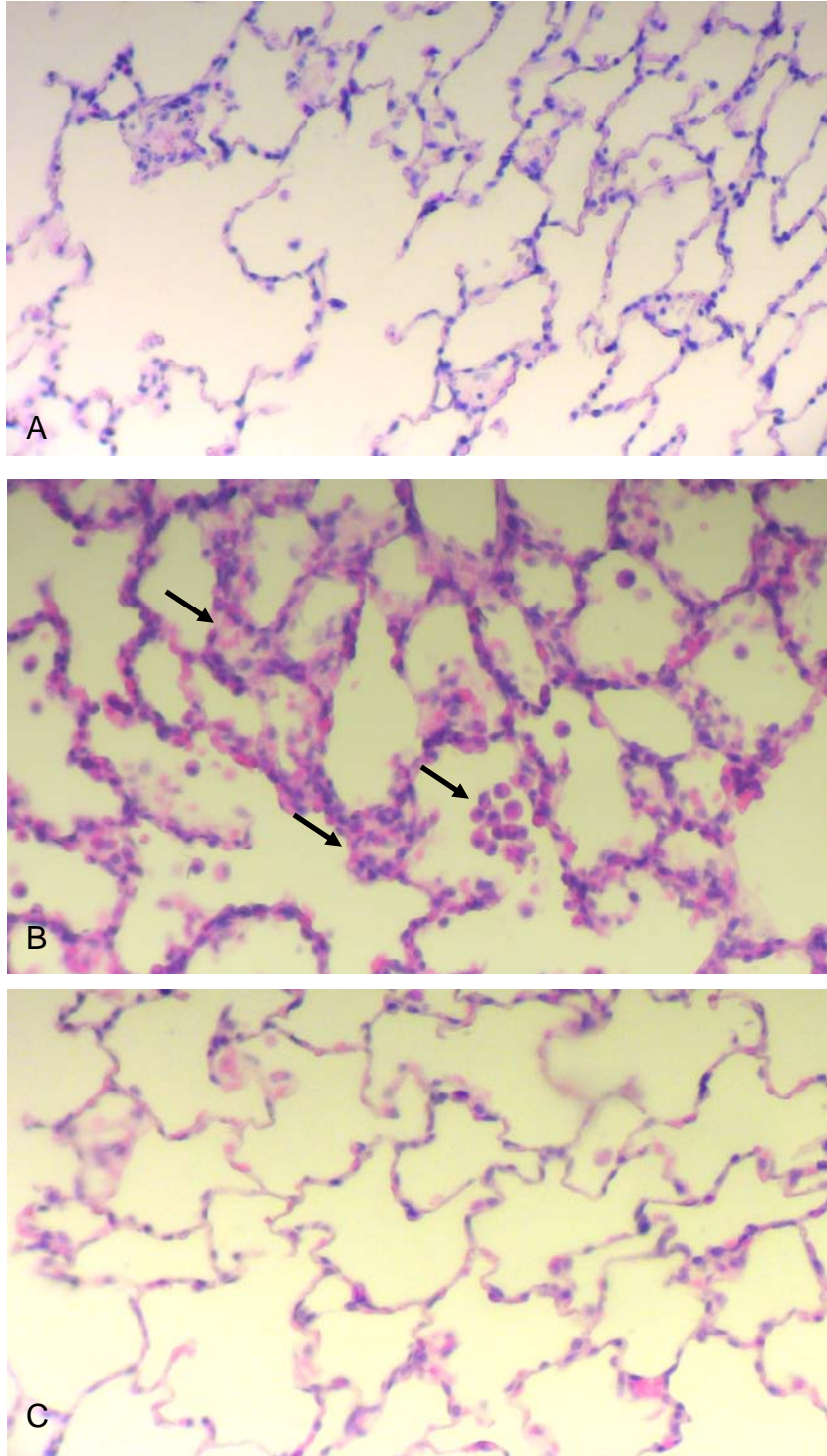


Figure 6.2.3.2: H&E staining of lung tissue from Saline (SAL) treated rabbit (A), SAL-LPS treated rabbit (B), and GC-LPS treated rabbit (C). More septal heterophils are present in LPS treated rabbits. Arrows show macrophages and neutrophils. Original magnification: 10.

Haematoxylin and Eosin Staining of Liver Tissue:

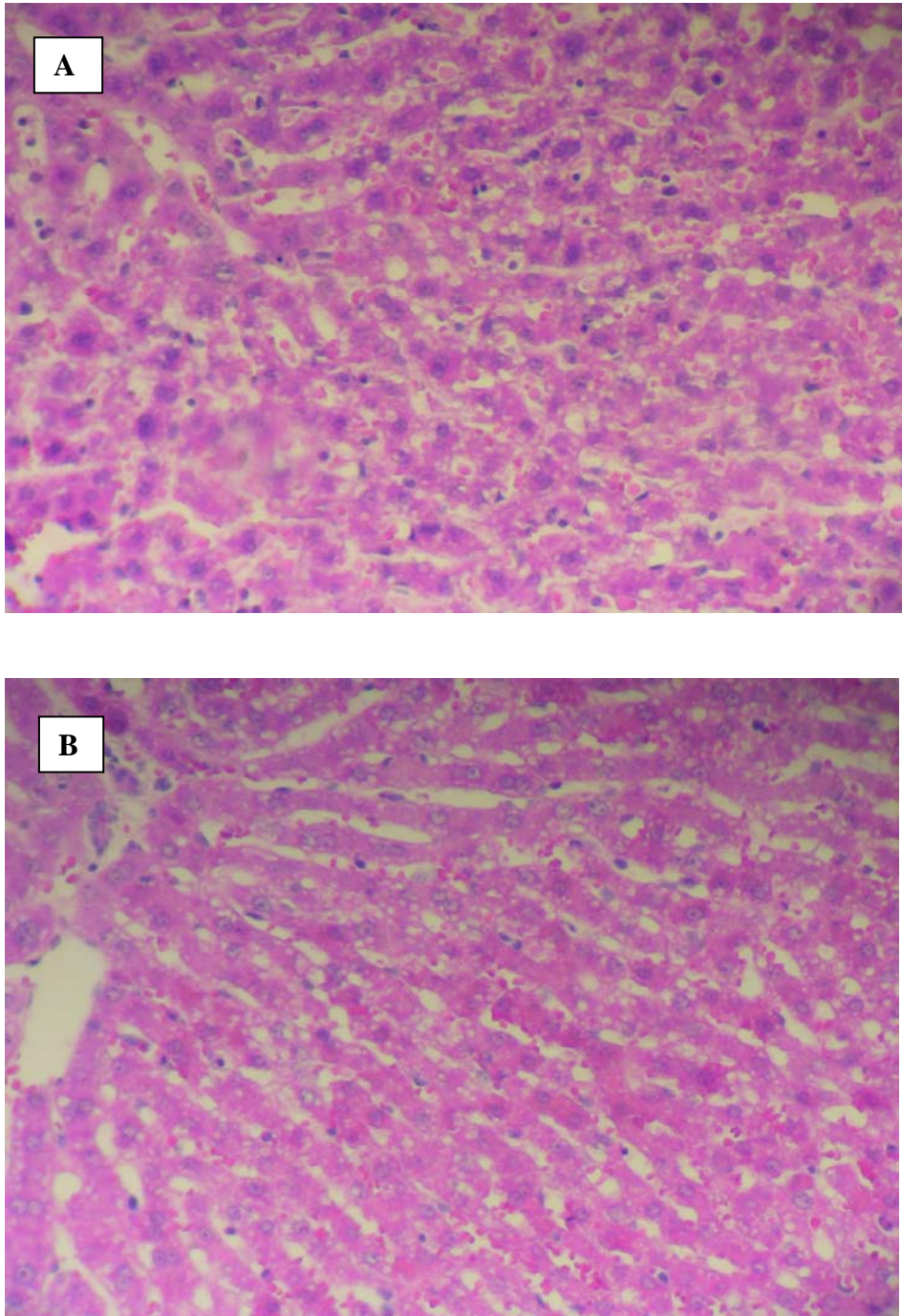


Figure 6.2.3.3: H&E stain of liver tissue from saline (A) and GC treated rabbits (B) after receiving LPS. Note heterophil infiltration in liver of SAL-LPS rabbit. Original magnification: 10.

Myeloperoxidase Assay from Lung Tissue:

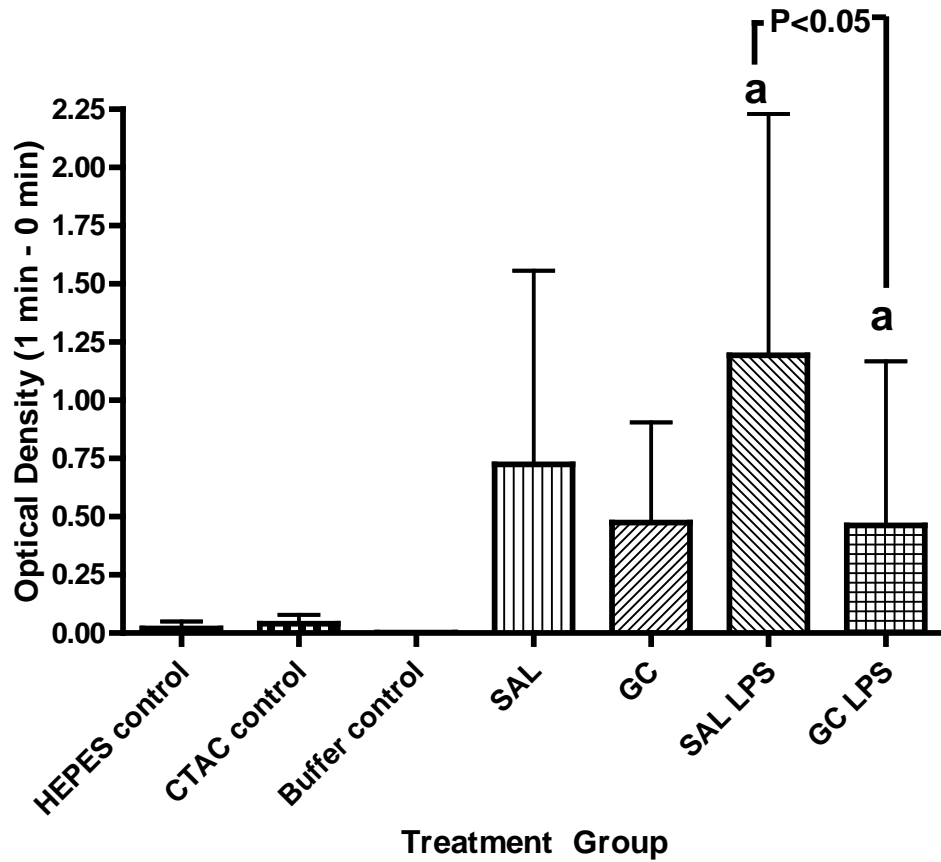


Figure 6.2.5: Mean \pm SD (n=6 each group) of myeloperoxidase concentrations in lung tissue from rabbits treated with either saline or GC with or without LPS. Pooled data from three analyses.

a indicates significant difference from each other ($P < 0.05$)

ELISA Data: BAL Fluid; TNF α :

A.

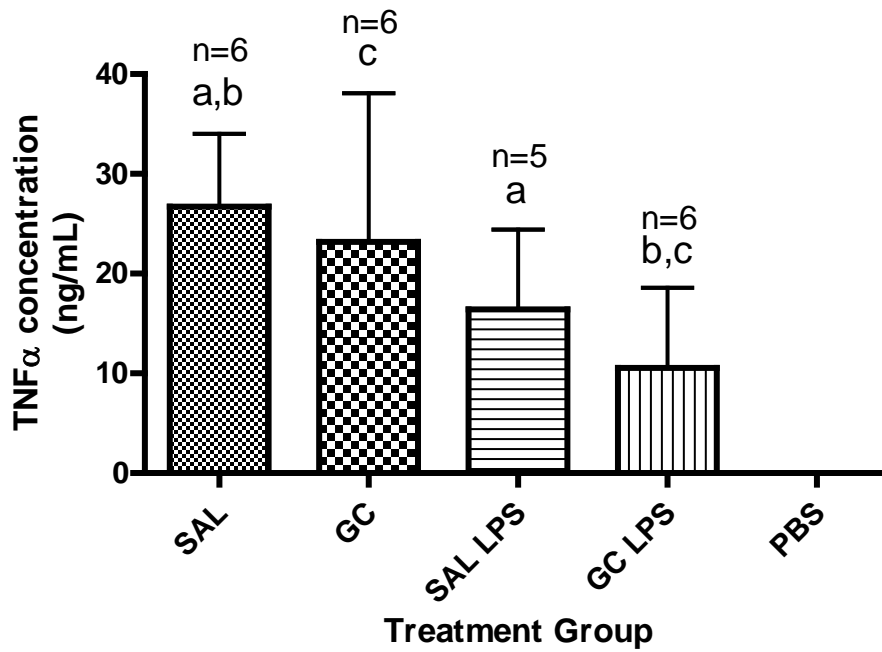
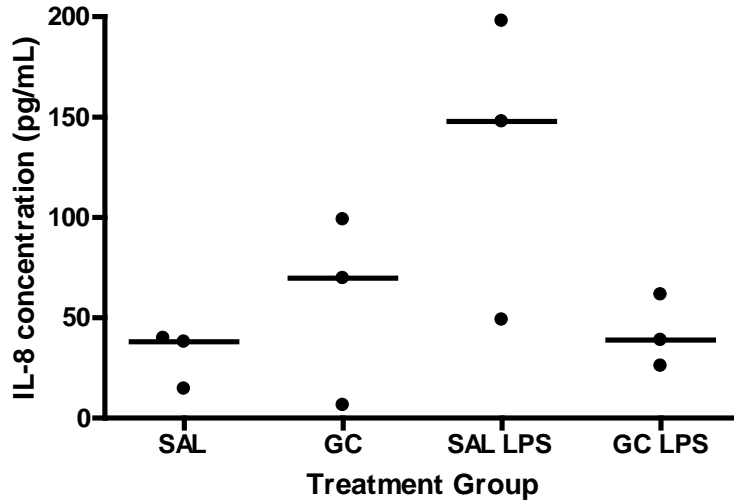


Figure 6.2.6.1: TNF α concentrations in BAL fluid (A) from control rabbit lungs and rabbits receiving LPS. Results from three analyses (mean \pm SD).

Same letter indicates significant difference between groups designated with that letter

ELISA Data: BAL Fluid; IL-8 and MCP

B.



C.

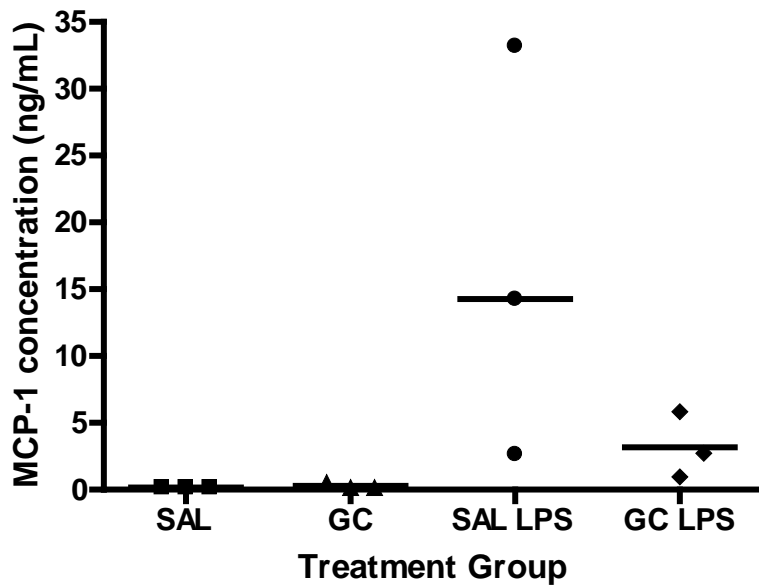


Figure 6.2.6.1: IL-8 (B) and MCP-1 concentrations in BAL fluid (C) from control rabbit lungs and from rabbits receiving LPS. Bars represent median values. No significant difference (Kruskall-Wallis test: B ($P=0.2$); C ($P=0.024$ before Dunn's test))

Serum TNF α concentration:

A.

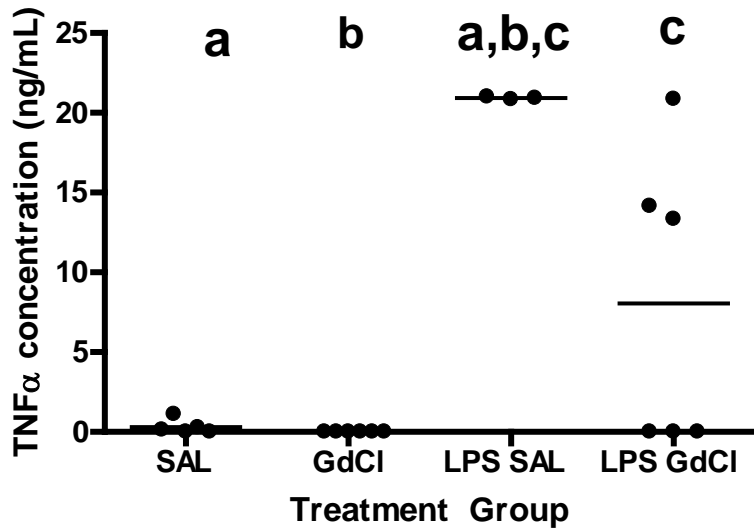
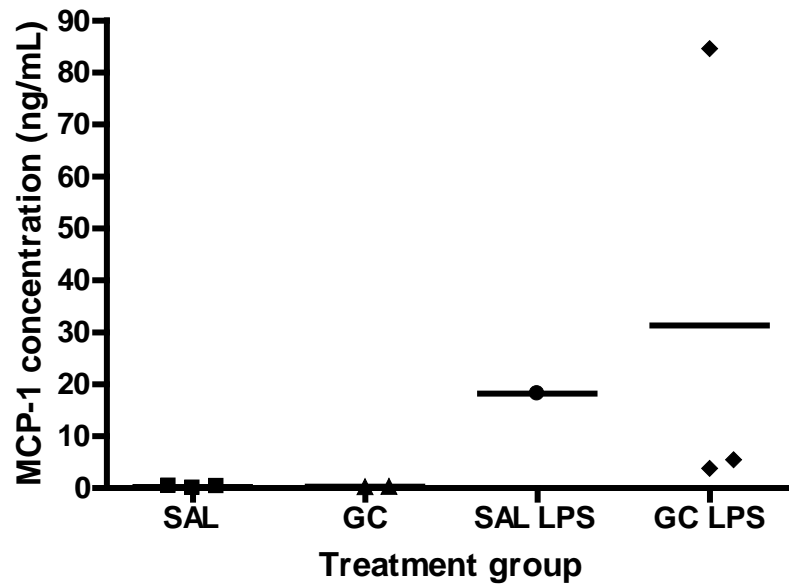


Figure 6.2.6.2: TNF α concentrations in serum (A) from control rabbits and rabbits receiving LPS. Bars represent mean values. Significant difference between groups SAL and SAL-LPS ($P < 0.001$); GC and SAL-LPS ($P < 0.001$); SAL-LPS and GC-LPS ($P < 0.05$) (1-way ANOVA test with Bonferroni post-hoc correction).

Same letter over a group indicates statistical difference from each other.

Serum MCP-1 and IL-8 concentration:

B.



C.

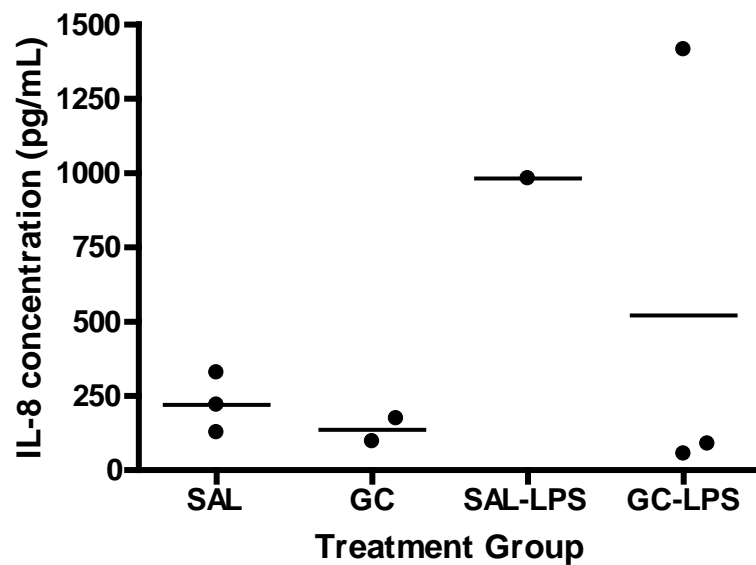
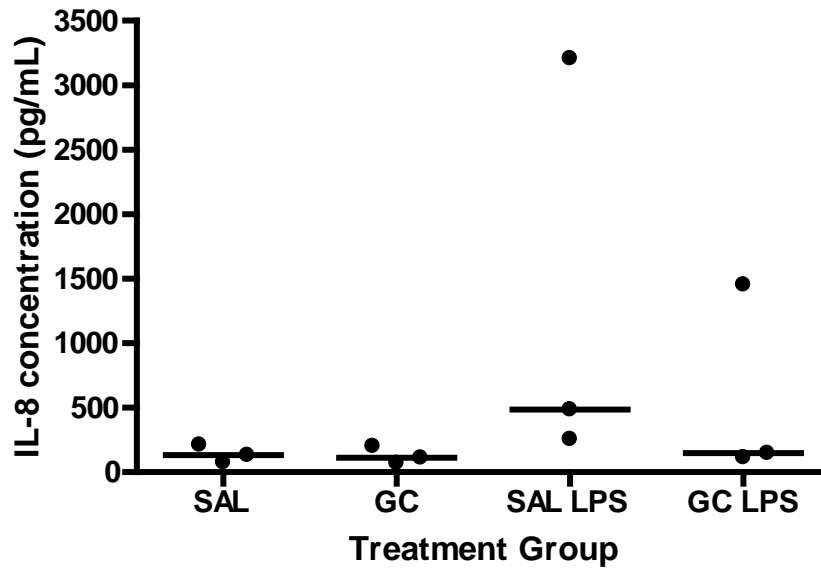


Figure 6.2.6.2: MCP-1 (B) and IL-8 (C) concentrations in serum from control rabbits and rabbits receiving LPS. Bars represent median values. No significant difference. (Kruskall-Wallis test: B ($P=0.1$); C ($P=0.6$))

Lung and Liver IL-8 concentration:

A



B

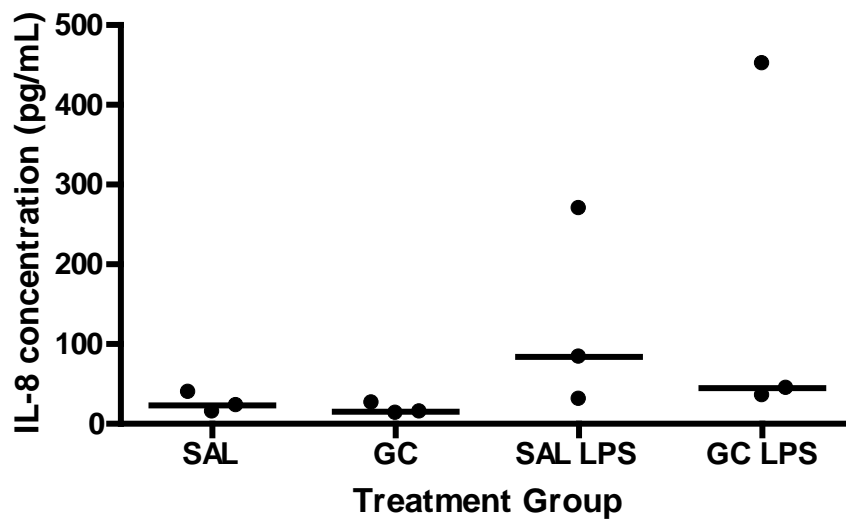
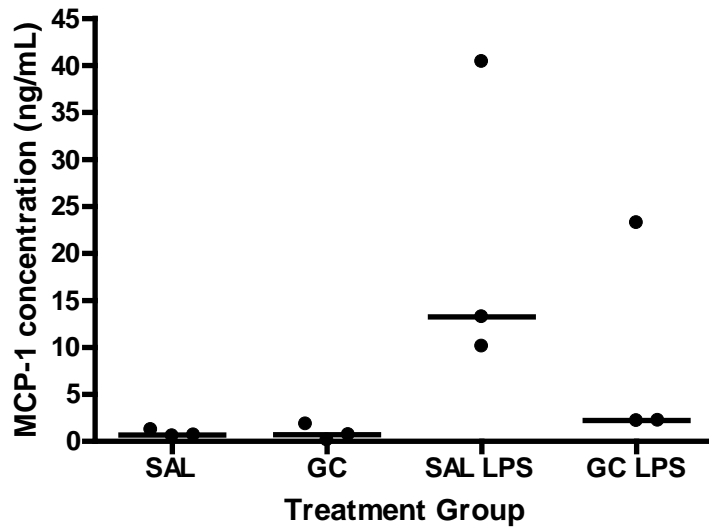


Figure 6.2.6.3: IL-8 concentrations in lung (A) and liver (B) tissue from control rabbits and rabbits receiving LPS. Bars represent median values. No statistical significance found (Kruskall-Wallis test: A ($P=0.12$); B ($P=0.066$))

Lung and Liver MCP-1 concentration:

C.



D.

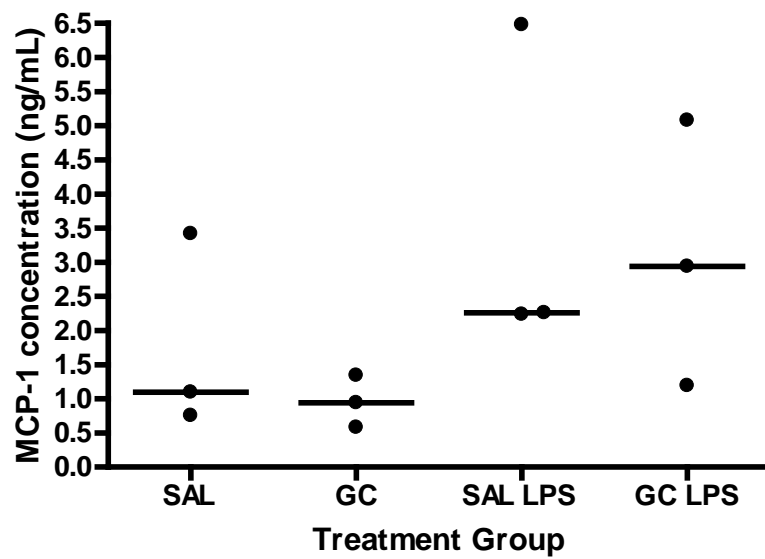
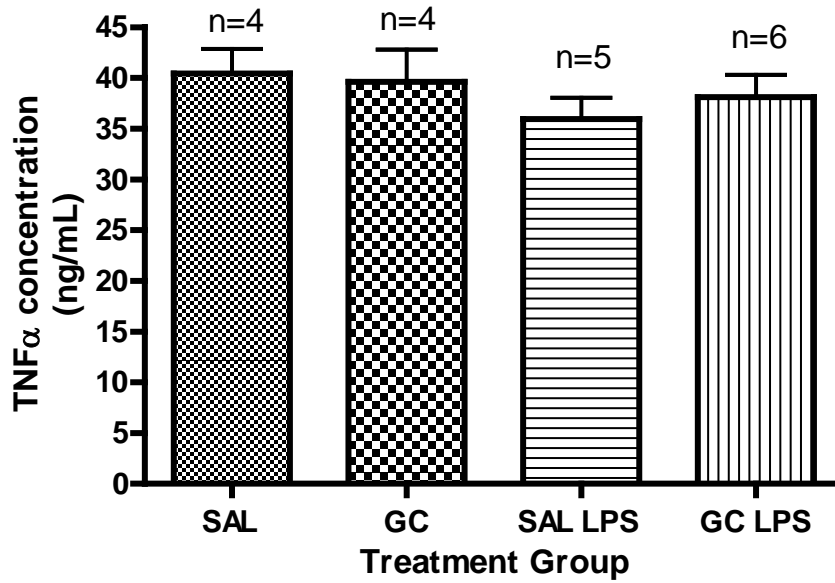


Figure 6.2.6.3: MCP-1 concentrations in lung (C) and liver (D) from control rabbits and rabbits receiving LPS. Bars represent median values. No statistical differences (Kruskall-Wallis test: C ($P=0.035$ before Dunn's test); D ($P=0.18$))

Lung and Liver TNF α concentration:

E.



F.

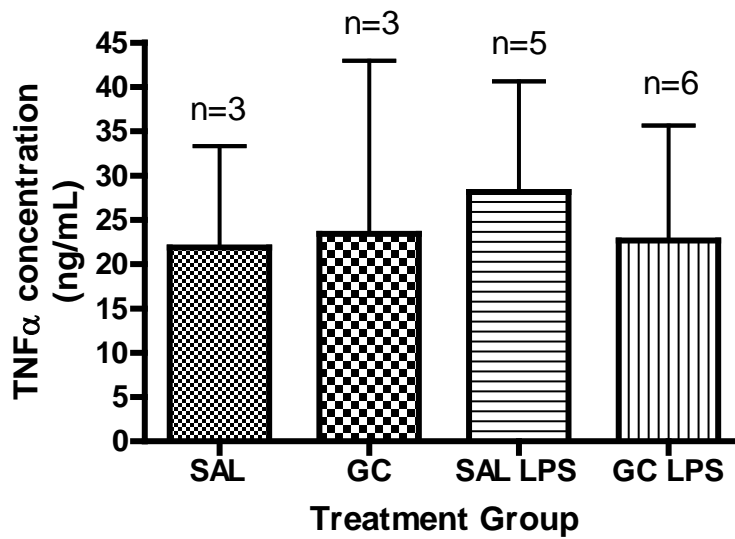
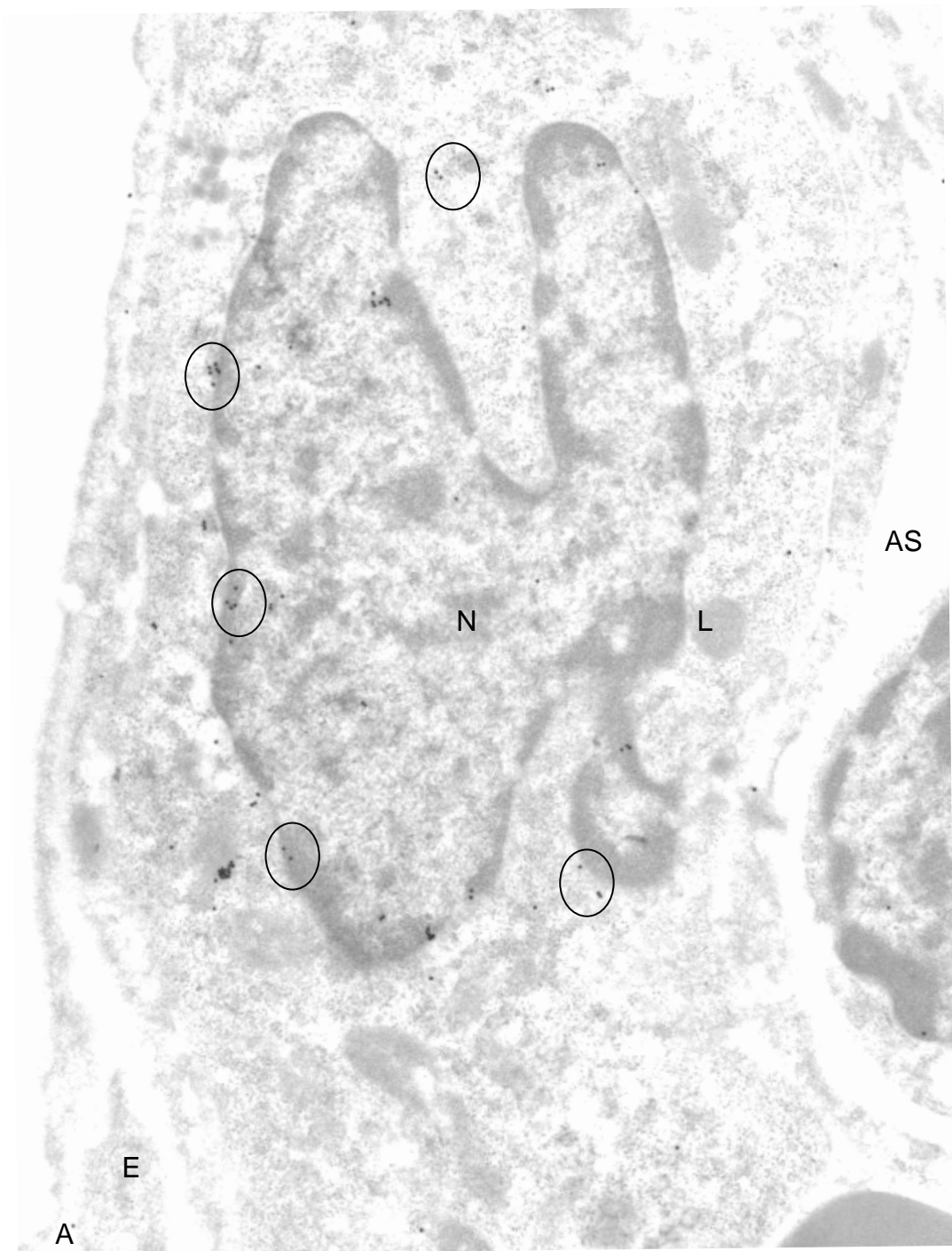


Figure 6.2.6.3: TNF α concentrations (mean \pm SD) in lung (E) and liver (F) from control rabbits and rabbits receiving LPS. Pooled data from three analyses.



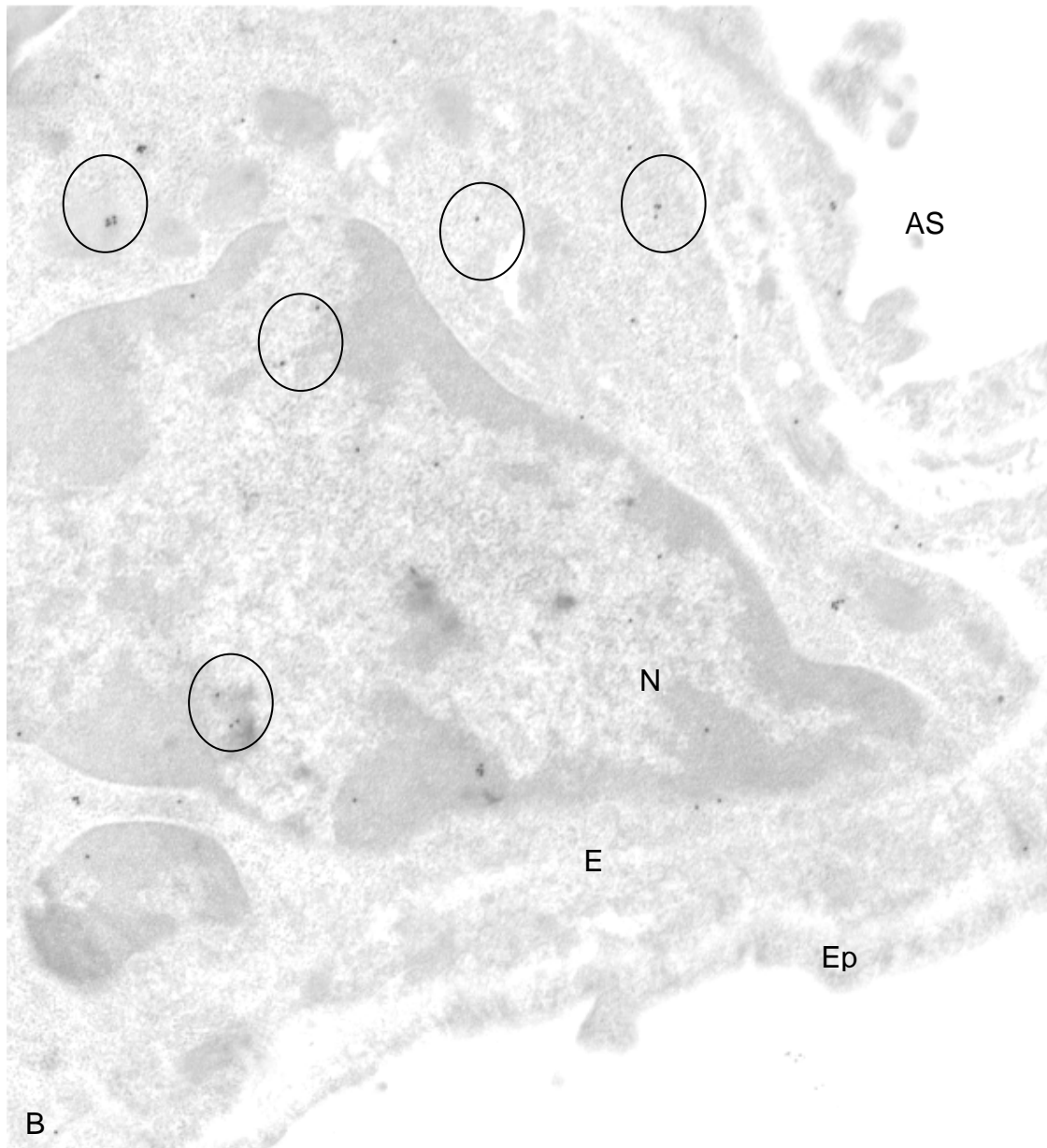


Figure 6.2.6.4: Immunostaining for TNF α : Lung section from SAL (A) and SAL-LPS-treated (B) rabbit show staining for TNF α (circles) in cytoplasm and nucleus (N) of PIMs. L: Lysosome; E: Endothelium; Ep: Epithelium; AS: Alveolar Space; Original magnification: 13000

RT-PCR Data:

RT-PCR TNF α mRNA

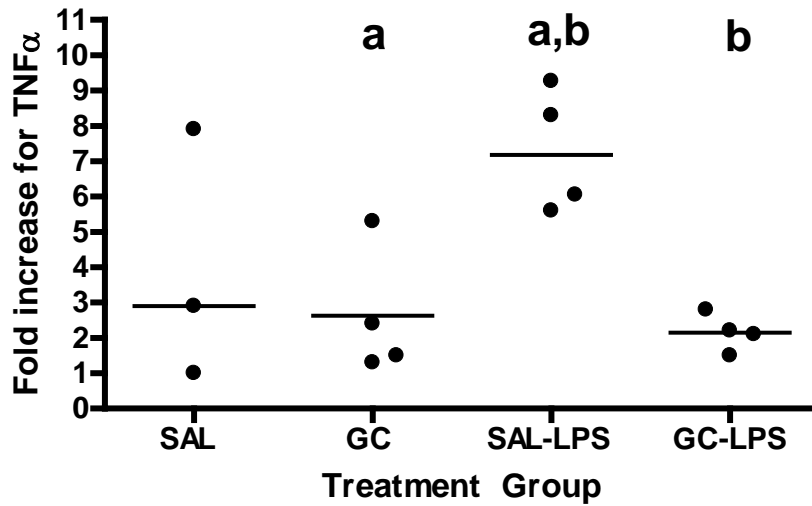


Figure 6.2.7.1: RT-PCR data for TNF α mRNA in control rabbit lungs, and saline or GC pretreated and receiving LPS. Bars represent mean values. Statistical difference between SAL-LPS and GC ($P < 0.05$) and between SAL-LPS and GC-LPS ($P < 0.05$) using 1-way ANOVA with Bonferroni post-hoc test.

Same letter represents significant difference from each other ($P < 0.05$)

RT-PCR IL-1 β :

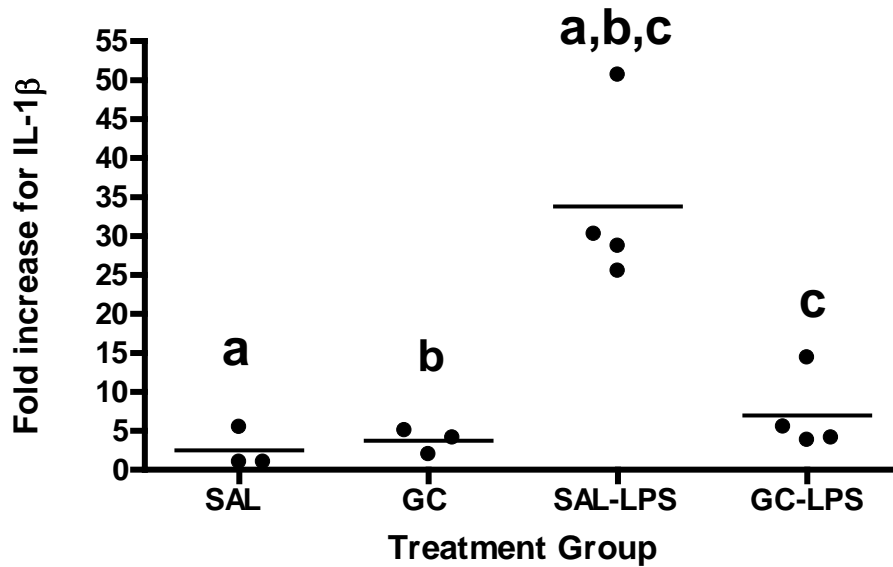


Figure 6.2.7.2: RT-PCR data for IL-1 β mRNA in control rabbit lungs, and saline or GC pretreated and receiving LPS. Bars represent mean values. Statistical difference between groups SAL and SAL-LPS ($P < 0.001$); GC and SAL-LPS ($P < 0.01$) and SAL-LPS and GC-LPS ($P < 0.01$) using 1 way ANOVA with Bonferroni post-hoc test.

Same letter represents significant difference from each other ($P < 0.05$)

RT-PCR IL-6 :

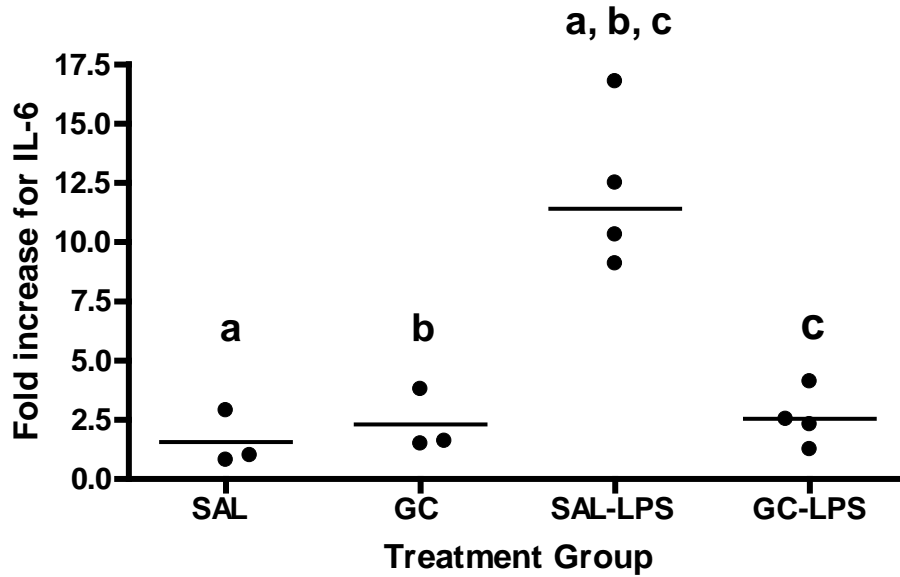


Figure 6.2.7.3: RT-PCR data for IL-6 mRNA in control rabbits, and saline or GC pretreated rabbits receiving LPS. Bars represent mean values. Statistical difference between groups SAL and SAL-LPS ($P < 0.001$); GC and SAL-LPS ($P < 0.001$) and SAL-LPS and GC-LPS ($P < 0.001$) using 1 way ANOVA with Bonferroni post-hoc test.

Same letter represents significant difference from each other ($P < 0.05$)

7. DISCUSSION

7.1 Search for PIMs in normal rabbit lung

The microscopic data revealed PIMs in normal rabbits. Large mononuclear phagocytes in the lung septa of normal rabbits were observed with routine light microscopy on paraffin-embedded and epoxy-embedded lung sections, and routine electron microscopy revealed intravascular macrophages attached to the lung capillary endothelium. Taken together, these data demonstrate PIMs are present in normal rabbits. No morphometric comparisons were performed, so conclusions on the density of PIMs in the rabbit compared to sheep or horse is difficult. The relatively lower density of PIMs in rabbits and the dominance of Küpffer cells in particle removal may have led to earlier controversial conclusions that rabbits lack PIMs (Brain *et al.*, 1999), (Horiguchi *et al.*, 1996). Identification of PIMs in this study may explain indirect evidence of PIMs in rabbit lungs of other reports (Carrasco, 1991). Humans are also reported not to have PIMs, but one report described their presence after examining normal parts of human lung from patients undergoing thoracotomy for noninfectious disease (Dehring and Wismar, 1989). Morphometric analysis was not performed to quantify PIM numbers, but Dehring and Wismar comment the density was lower than in sheep and pig lungs. It could be argued we were also observing monocytes normally present in the pulmonary circulation. Unlike Dehring & Wismar's study, we attempted to remove loosely bound monocytes during flushing of the pulmonary circulation after euthanasia, leaving tightly adhered PIMs. Furthermore, electron microscopy showed that the cells in question had ultrastructural features of macrophages and not of monocytes. Taken together, the data reveal PIMs in rabbits.

To determine presence of PIMs, the cells were depleted using GC in six rabbits. The final PIM count was compared between normal and GC-treated rabbits. Using electron microscopy, many apoptotic PIMs were observed, but there was no statistical decline in the numbers of PIMs in GC-treated rabbits ($P=0.2$). The numbers of PIMs in GC-treated rabbits may have been over-estimated because even the dysfunctional apoptotic PIMs would have still reacted with RAM-11 antibody as apoptotic cells maintain intact

membranes and their proteins. There is a species difference in the effect of GC on PIM numbers. Calves and horses treated with GC demonstrate significant reduction in PIM numbers at 48 hours and 72 hours, respectively (Singh *et al.*, 2004), (Parkhabar *et al.*, 2005). Therefore, it is possible that PIM numbers in GC-treated rabbits would have declined significantly at later time points. Nevertheless, some GC-mediated reduction in PIM numbers supports presence of PIMs in normal rabbits.

7.2 Impact of PIMs on endotoxin-induced inflammation

Blood sampling was difficult after injection of LPS because the rabbits were clinically debilitated. Mortality was increased in the SAL-LPS group. It appeared GC had a protective effect probably through limitation of inflammatory mediator release (TNF α , IL-1 β and IL-6), and prevention of high concentrations of toxic breakdown by-products from neutrophil (heterophil) phagocytosis (Hildebrand *et al.*, 2006). Systemic inflammatory response and high mortality has recently been reported in a rat model of PIM recruitment, and treatment with GC before infusion of endotoxin reduced lung inflammation, and inhibited mortality (Gill *et al.*, 2008). No post-mortem results were available, but circulatory collapse and coagulopathies from the inflammatory cascade could have led to death in SAL-LPS rabbits. Death from ALI appeared unlikely as surviving rabbits did not have severe ALI, although the lungs were not examined around the time death occurred (4-12 hours after LPS injection)

7.2.1 Effects on blood cells and metabolites

Intravenously injected LPS (250 μ g/kg) in rabbits is rapidly removed from the circulation by the liver, spleen and lung, with a calculated elimination half-life of 12 hours (Mathison and Ulevitch 1979). Endotoxin treatment also causes a significant reduction in peripheral total leukocyte numbers because of cells trapped in organs such as liver and lung (Reutershan and Ley, 2004), also observed in the current study. There appeared to be a reduction in white cell count in the SAL-LPS group compared to groups SAL and GC, but there was no statistical difference. A reduction in blood heterophil number between groups was not obvious, although LPS causes heterophil reduction (Cöl *et al.*, 2005).

The LPS-treated rabbits developed a prominent fully-compensated metabolic acidosis similar to a previous report at 10 hours after the same dose of LPS (Jesmin *et al.*, 2004). Stabilization of plasma pH within normal limits occurred through increased minute ventilation and lowered PaCO₂ and was also found in Jesmin *et al.*'s study. During sepsis, excessive hydrogen ions (metabolic acidosis) arise from poor oxygen delivery to cells and lactate production (Jesmin *et al.*, 2004). Stress hyperlactaemia also occurs from the respiratory burst in activated phagocytic cells (Mizock, 2000). Lactate was found to be elevated in all groups in our study, with no differences between groups. Normal lactate concentration in dogs and cats is <2.0 mmol/L, and this is probably true for rabbits (Pang and Boysen, 2007). It is unknown why control rabbits had high concentrations of lactate. Stress of handling has been shown to increase lactate concentrations in stress-susceptible pigs, and restraint may have increased lactate levels in all rabbits, including the SAL and GC control groups, despite attempts made to accustom rabbits to their test surroundings (Spencer, 1994). The rabbits receiving LPS tended to have higher chloride ion concentration. As anion gap and potassium concentration did not change appreciably between groups, the increased chloride may be due to early renal failure or circulating volume contraction (Koch and Taylor, 1992). Renal failure can contribute to the observed metabolic acidosis, but like lactate, increases anion gap due to retained sulphates and phosphates (Koch and Taylor, 1992).

Glucose and calcium ion concentrations tended to decrease in both groups receiving LPS compared to control. This response is typical of endotoxaemia and has been previously reported (Aderka *et al.*, 1987). Hypocalcaemia during sepsis is found in humans, horses, rats and pigs (Holowaychuk and Martin, 2007). Decrease in ionized calcium may be multi-factorial, and includes: parathyroid hormone imbalance, increased urinary loss of calcium, calcium chelation, tissue or cellular accumulation of calcium, hypomagnesaemia, and elevations in calcitonin precursors (Holowaychuk and Martin, 2007). Hepatic dysfunction during endotoxaemia leads to impaired glucose release and hypoglycaemia, complicated by a cytokine-induced increased peripheral glucose uptake into cells, especially within macrophages and neutrophils (Mizock *et al.*, 2000).

Serum TNF α concentrations in response to LPS challenge were higher in rabbits of the SAL-LPS group compared to the SAL, GC and GC-LPS groups. TNF- α levels have been found to peak at three hours after injection of LPS and remained elevated up to 10 hours post-LPS (Jesmin *et al.*, 2004). Another study revealed peak TNF α concentrations at two hours after 100 μ g/kg intravenous LPS, and levels declined to below detection by their last six hour sampling time-point (Rodriguez-Wilhemi *et al.*, 2003). There are very few reports documenting the concentrations of serum or plasma cytokines at 24 hours after LPS injection. TNF α concentration at 24 hours after 1 μ g/kg LPS was found to be within detectable limits, and approximated 10% of the concentration obtained at the three hour time-point (Arras *et al.*, 1996). Therefore, it is feasible that TNF α concentrations could still be detectable at 24 hours after LPS, especially as a higher dose was used. As occurred with this study, removal of intravascular macrophages including PIMs by GC has been shown to reduce TNF α concentrations in the circulation after LPS challenge in other species (Gong *et al.*, 2002).

Serum IL-8 concentrations were not found to be different between groups, but the statistical analysis was complicated by the presence of only one value in the SAL-LPS group because of limited sample. Plasma IL-8 concentrations have been found to increase early after LPS injection and were elevated at 6 hours after injection of the same dose of LPS (Rodriguez-Wilhemi *et al.*, 2003). It appears IL-8 and heterophil attraction is important for the production of MCP-1 (Miyazaki *et al.*, 2000). It might be expected that MCP-1 concentrations would remain high at 24 hours after LPS injection as the inflammation moves into resolution phase characterized by the recruitment of monocytes. This increase was observed in our study, but only in GC-LPS rabbits. Lack of samples was probably a factor in accurate assessment of conditions in the SAL-LPS group. MCP-1 can also be secreted by hepatic stellate cells in response to LPS, and may have been the source of MCP-1 in intravascular macrophage depleted rabbits (Ramadori *et al.*, 2008).

7.2.2 Effects of LPS on the Lung

Injection of LPS did not cause severe ALI in these rabbits compared to previous reports using 100 or 500 μ g/kg intravenous LPS with euthanasia at 10 and 8 hours, respectively

(Jesmin *et al.*, 2004), (Zhijun *et al.*, 2003). The different sampling times make direct comparisons between studies difficult. We chose to euthanize the rabbits at 24 hours after LPS injection to assess survival over a longer term. A decrease in PaO₂ at 10 hours after LPS injection is reported elsewhere, whereas PaO₂ in our LPS treated rabbits increased slightly (Jesmin *et al.*, 2004). PaO₂ may have been enhanced by increased alveolar O₂ levels produced by hyperventilation and lowered alveolar CO₂ levels. A more accurate examination of oxygenation using A-a O₂ difference also found no changes. However, conclusions are difficult to draw as there were few arterial blood samples available in each of the GC-LPS and SAL-LPS groups. Examination of the wet/dry ratio did not reveal any differences between groups indicating that lung oedema was not prominent in our model, however a complicating factor may be the use of lung perfusion and retention of water within capillaries. Increases in wet/dry ratio at 10 hours after LPS injection have been reported along with increased movement of radio-labelled albumin into the alveoli (Jesmin *et al.*, 2004). Further examination of histological sections of lung and liver by a pathologist blinded to treatment group would have further strengthened the study.

White cell count and differential in BAL fluid cytospin was not statistically different between any groups. No migration of heterophils into the alveolar space was observed despite higher concentrations of IL-8 in BAL fluid of group SAL-LPS. The septal heterophil count was not different between groups receiving LPS, but significantly higher than found in lung septae of normal rabbits. Interestingly, the MPO assay revealed more activated heterophils in the SAL-LPS rabbit lungs compared to GC-LPS lungs.

Neutrophil (heterophil) recruitment is a complex process which involves cytokine-induced activation of vascular endothelium and expression of adhesion molecules such as selectins and integrins ((Kuebler , 2005). The role of cytokines such as TNF α , IL-1 β and IL-6, which are involved in the early phase of acute lung inflammation, is highly important in starting the process of leukocyte recruitment. GC treatment before LPS infusion especially reduced the concentrations of IL-8 and MCP-1 protein, as well as, TNF α , IL-1 β and IL-6 mRNA in lung tissue and BAL fluid compared to SAL-LPS rabbits. The lung endothelium of the GC-LPS rabbits may be less activated compared to endothelium in the SAL-LPS group, possibly through removal of local stimulatory

sources such as PIMs. Lung injury and MPO activity were reduced in an ischaemia-reperfusion isolated rabbit lung model after removal of alveolar macrophages with GC (Gazoni *et al.*, 2007). The removal of alveolar macrophages reported in that study conflicts with evidence that intravenous administration of GC only affects intravascular macrophages (Hildebrand *et al.*, 2006), (Singh and de la Concha-Bermejillo, 1998). It is possible Gazoni *et al* were removing PIMs from their isolated rabbit lungs and not alveolar macrophages.

A recent study performed in rabbits removed intravascular macrophages using liposome encapsulated clodronate, and were then administered *Pneumococcus aeruginosa* via the trachea (Kurahashi *et al.*, 2009). The study proved loss of macrophages through examination of carbon particle uptake by the liver and spleen. The study did not examine carbon particle uptake in the lungs therefore the contribution of PIMs was not known. That study did find however, similar to our study, that only intravascular macrophages were removed as there was no difference in RAM-11 positive alveolar cells between depleted rabbits and control group (Kurahashi *et al.*, 2009). Using this bacterial model of lung injury, depletion of macrophages resulted in more lung injury. This may be a result of uncontrolled bacterial invasion within the lungs and movement of bacteria into the circulation, whereas our model used intravenous endotoxin. Presence of PIMs in these two scenarios could explain the different results observed.

Elimination of other intravenous macrophages such as Küpffer cells by GC has been found to reduce lung injury in a rat model which normally do not have PIMs (Okutan *et al.*, 2004), (Tullis *et al.*, 1996). Removal of other intravascular macrophages could explain reduced lung injury in our study, although removal of the Küpffer cells did not appear to reduce liver injury in our model. Subjective evaluation of liver tissues in this study showed lack of effect of GC on Küpffer cells. Although we cannot prove with our data, PIMs could have predominantly been affected by GC in our rabbits by removing GC during its first pass through the lungs with administration using a marginal ear vein. It has been previously reported that GC mainly depletes PIMs, and not Küpffer cells, when administered through a jugular vein in calves with PIMs (Singh *et al.*, 2004),

although other groups have found that GC can still remove Küpffer cells in other species (Szabo *et al.*, 2002).

Mathison and Ulevitch (1979) examined the effects of LPS (*E.coli* O111:B4 LPS) and LPS (*Salmonella minnesota* R595), and found the former LPS type produced some accumulation of monocytes and neutrophils within the lung, but the R595 form produced high concentrations of these cells heavily labelled with LPS within five minutes of injection. Therefore, the final effect of intravascular macrophages and LPS on ALI can also depend on the type of LPS used, as well as dose used and route of administration of LPS, and choice of examination time-point. Increased MCP-1 concentrations in serum and lung tissue in LPS treated rabbits could have attracted more monocytes/macrophages to the pulmonary circulation by 24 hours after LPS injection (Ohgami *et al.*, 1992). The pulmonary circulation was thoroughly flushed during lung tissue collection and loosely bound monocytes should have been removed.

The high TNF α concentrations found in BAL fluid, lung and liver tissue of normal rabbits is difficult to explain. Concentration of TNF α in normal individuals is usually below detectable values (Arras *et al.*, 1996). From examination of ELISA technique, it did not appear this source of error was the cause. Long-term storage may have had an effect on TNF α concentrations. The samples had been stored for three years at -80°C before analysis. Using a mathematical model, 90% recovery of TNF α protein is possible when samples are stored at -70°C for up to five years (Ruiz *et al.*, 1996). It appears logical that TNF α concentrations should slowly decrease over time at low temperatures, and not increase. No studies were found which examined TNF α stability with other tissues or fluids at -80°C for as long as three years. Other investigators have examined whole and separated blood, and found TNF α concentrations can increase by 9.6% if incorrect handling of blood samples is used, and IL-6 can decrease by 14.3% (Flower *et al.*, 2000). TNF α concentrations can also increase by 17% with three freeze-thaw cycles (Flower *et al.*, 2000). EDTA was found to be the most stable anticoagulant for use with cytokine studies, and was used in our study (Flower *et al.*, 2000). The rabbits were all handled one hour before study procedures to apply EMLA (prilocaine/lidocaine) cream to

their ears. Restraint has been found to decrease TNF α concentrations by increasing IL-6 levels, and lidocaine decreases TNF α concentrations, therefore these procedures do not explain the results found (Hale *et al.*, 2003), (Mikawa *et al.*, 1994).

It is also expected to find low TNF α concentrations in lung lavage fluid of rabbits receiving IV LPS because the alveolar epithelial and capillary endothelium acts as a barrier compartmentalizing cytokines, and directing the inflammatory response to the correct area (Kuebler, 2005). In isolated lung preparations, LPS in perfusate has been found to increase lung lavage TNF α concentration in a time-dependent manner, and levels increased to 0.6 ng/mL by three hours, but were less than 15% of the perfusate TNF α concentration (Ghofrani *et al.*, 1996). In our study, BAL TNF α concentrations are very similar to the serum TNF α concentrations in rabbits of the same group. Ghofrani *et al.* (1996) used lower doses of LPS compared to our study and this may account for their lower concentrations of TNF α in lung lavage fluid and different time-points were used. The study was also performed in an isolated lung preparation and the perfusate did not contain neutrophils which are important in ALI (Ghofrani *et al.*, 1996). Alveolar macrophages are a potential source of TNF α (Laskin *et al.*, 2001), but it is unclear how these isolated cells would become activated by IV LPS. There was no evidence of capillary leak in our study, and heterophils were not observed in BAL cytospin preparations. In intact rabbits, it was found that TNF α and IL-8 concentrations increased in lavage fluid at eight hours following injection of a high dose (500 μ g/kg) of IV LPS, but there was strong evidence of capillary leak in that study (Zhijun *et al.*, 2003). Very high doses of IV LPS (5 mg/kg) in rabbits produced high mortality within six hours, and movement of heterophils into the alveolar space with high concentrations of IL-8 in BAL fluid (Kwak *et al.*, 2004). Compartmentalization of the effects of intratracheal or IV LPS does not appear to hold true when high doses of LPS are used, and there is breakdown of the alveolar-capillary barrier.

7.2.3 Effects of LPS on the Liver

After euthanasia, in all rabbits receiving LPS, gross examination revealed the liver to be light coloured and swollen. Histological examination revealed heterophil infiltration in

both groups receiving LPS. The liver contains the largest mass of intravascular macrophages in most species, and this is probably true of rabbits since the number of PIMs is limited. Therefore activation of these macrophages can have endocrine effects on the rest of the body through release of cytokines and other inflammatory mediators (Szabo *et al.*, 2002). In rabbits, LPS localizes in Küpffer cells after injection (Mathison and Ulevitch, 1979). In rats, when Küpffer cells are blocked by GC, the hepatocytes remove endotoxin (Szabo *et al.*, 2002). The excretion of LPS then occurs through bile which completely inactivates endotoxin. Otherwise, removal of LPS is facilitated through LPS binding proteins and formation of high and low density micelles (Szabo *et al.*, 2002). In rats without PIMs, it has also been found that removal of Küpffer cells will increase ALI and increase inflammatory mediators, but this was not found to be the case in our rabbits (Kono *et al.*, 2008). In our study, removal or block of intravascular macrophages decreased mortality from LPS injection. Redirection of LPS through hepatocytes, and the reduction in large quantities of inflammatory mediators produced from Küpffer cells could have reduced the remote detrimental effects leading to haemodynamic instability and death. IL-8 can be produced by hepatocytes in place of Küpffer cells, and may explain a higher concentration of IL-8 in the liver of one GC-LPS rabbit (Ramadori *et al.*, 2008). As discussed earlier the reduced mortality, expression of inflammatory mediators and accumulation of neutrophils (heterophils) in the lung may largely be due to the effect of GC on PIMs, and not indirectly inhibition of Küpffer cells and their remote actions on the lungs (Singh and de la Concha-Bertejillo, 1998).

The liver appeared to contain high concentrations of TNF α , but there were no differences between groups. It appeared GC did not moderate TNF α production in the liver. IL-8 was also elevated in one rabbit of the GC-LPS group, and neutrophils were found within the liver on histological examination in both groups. MCP-1 concentrations in the liver were also similar between groups receiving LPS. Further examinations of the liver such as MPO assay, liver RT-PCR of pro-inflammatory cytokines, and wet/dry ratios may have helped to clarify whether GC had a liver-sparing effect. Examination of existing data indicates GC did not appear to protect the liver in these rabbits.

GC is a potent inhibitor of macrophage phagocytosis and induces apoptosis of these cells by blocking K-type Ca^{2+} channels, and competing with membrane calcium since their radii are similar (Palasz and Czekaj, 2000). It is also a cytochrome p450 inhibitor and can reduce the production of toxic free radicals, possibly through a paradoxical increase in TNF and IL-6 production, or decreased IL-4 production. Its affects are predominantly on intravascular macrophages, and does not appear to affect alveolar and splenic macrophages (Hildebrand *et al.*, 2006).

Damage to various organs can also be altered depending on point of entry of LPS into the body. If the liver receives the bulk of the LPS as first pass, it will remove the LPS and spare other organs such as the lung (Shimada *et al.*, 2006). The rabbits in this study received LPS through a marginal ear vein and this avoids the hepatic first pass effect.

8. CONCLUSIONS

It appears that rabbits do contain a few PIMs and could be considered intermediate between species with no confirmed PIMs such as rats, and those that obviously have these cells such as horses. Elimination of other intravenous macrophages such as Küpffer cells did not appear to have an effect on liver injury, probably as hepatocytes dealt with LPS removal and produced cytokines. Removal of PIMs appeared to reduce ALI in the rabbit.

9. PROPOSED FUTURE RESEARCH

It was hoped that the rabbit could be used as a convenient model for research into equine ALI and the effects of treatments such as inhaled anaesthetics, however this model is not an appropriate reflection of the equine lung, and another model such as the ovine species may be more suitable.

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